PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:
KREMER, Simon, M.
Mewburn Ellis
York House
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London WC2B 6HP
ROYAUME-UNI

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 29 March 2001 (29.03.01)	
Applicant's or agent's file reference SMK/LP5872353	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/GB00/03525	13 September 2000 (13.09.00)	17 September 1999 (17.09.99)

Applicant
PLANT BIOSCIENCE LIMITED et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AG,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EA,EE,EP,ES,
   FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,
   MN,MW,MX,MZ,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   29 March 2001 (29.03.01) under No. WO 01/21822

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

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Continuation of Form PCT/IB/308

NOTICE [redacted]ORMING THE APPLICANT OF THE CO[redacted]UNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year)	IMPORTANT NOTICE
29 March 2001 (29.03.01)	
Applicant's or agent's file reference	**International application No.**
SMK/LP5872353	PCT/GB00/03525

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted]B 00/03525

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12Q1/68 C07K14/415 C07K16/16 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, MEDLINE, BIOSIS, STRAND, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHANDLER ET AL: "arabidopsis mutants showing an altered response to vernalisation" PLANT JOURNAL,GB,BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 10, no. 4, 1996, pages 637-644, XP002139145 ISSN: 0960-7412 cited in the application the whole document ---	1-34
Y	SATO S ET AL: "A sequence-ready contig map of the top arm of Arabidopsis thaliana chromosome 3." DNA RESEARCH, (1999 APR 30) 6 (2) 117-21. ISSN: 1340-2838., XP000973646 figure 1 --- -/--	1-34

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 February 2001	19/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Burkhardt, P

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEVY YARON Y ET AL: "The transition of flowering" PLANT CELL,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 10, no. 12, December 1998 (1998-12), pages 1973-1989, XP002132682 ISSN: 1040-4651 the whole document	1-34
A	LIU YAO-GUANG ET AL: "Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 11, 25 May 1999 (1999-05-25), pages 6535-6540, XP002158766 May 25, 1999 ISSN: 0027-8424 the whole document	1-34
A	WILSON A ET AL: "ANALYSIS OF THE MOLECULAR BASIS OF VERNALIZATION IN ARABIDOPSIS THALIANA" SEMINARS IN CELL AND DEVELOPMENTAL BIOLOGY,GB,ACADEMIC PRESS, vol. 7, no. 3, 1996, pages 435-440, XP000609514 ISSN: 1084-9521 the whole document	1-34

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PATENT COOPERATION TREATY

REC'D 15 JAN 2002
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
SMK/LP5872353		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/03525	13/09/2000	17/09/1999
International Patent Classification (IPC) or national classification and IPC		
C12N15/82		
Applicant AIB		
PLANT BIOSCIENCE LIMITED		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/03/2001	11.01.2002
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Burkhardt, P Telephone No. +49 89 2399 7456

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/03525

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-73	as originally filed				

Claims, No.:

10 (part),11-21, 29 (part),30-34	as originally filed				
1-9,10 (part),22-28, 29 (part)	as received on	07/08/2001	with letter of	06/08/2001	

Drawings, sheets:

1/8-8/8	as originally filed				

Sequence listing part of the description, pages:

1-26, filed with the letter of 16.11.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☒ furnished subsequently to this Authority in written form.

   ☒ furnished subsequently to this Authority in computer readable form.

   ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☒ The statement that the information recorded in computer readable form is identical to the written sequence

listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1 - 34
	No:	Claims	
Inventive step (IS)	Yes:	Claims	2 - 34
	No:	Claims	1
Industrial applicability (IA)	Yes:	Claims	1 - 34
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I

Basis of the opinion

The amended claims filed with the letter of 06.08.2001 are formally acceptable under Article 34(2)(b) PCT.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The following documents (D) are referred to in this report; the numbering is following the order of the International Search Report:

D1 Chandler *et al.*, 1996. Plant J. 10:637-644
D2 Sato *et al.*, 1999. DNA Res. 6:117-121.

1. Article 33(2)(3) PCT (Novelty and inventive step)

1.1 Present claim 2 is directed to an isolated nucleic acid sequence (SEQ ID NO:11) encoding a protein (VRN1) that alters the vernalisation response in *Arabidopsis*. The nucleic acid sequence and the protein appear to be novel over the prior art presently available to the IPEA.

1.2 The closest prior art D1 discloses a F2 population of a cross between a *vrn1-1* and a *fca* plant. The F2 plants were used to localise the *VRN1* locus in *Arabidopsis thaliana*. The locus was mapped on chromosome 3 between RFLP markers *mi207* and *mi399* (page 649, paragraph bridging first and second column).

1.3 The present application, however, found the *vrn1* locus outside this range. Thus, it appears that the cloning of the gene was not straight forward and an inventive step can be recognised. Present claim 2 and dependent claims 2 - 8 as well as for claims 9 - 34 relating to parts of the *vrn1* sequence, vectors containing the sequence, methods of isolating the sequence, vectors and cells containing the sequence, methods of influencing the vernalisation phenotype and the *vrn1*

promoter meet the requirements of Article 33(2)(3) PCT.

1.4 Claim 1, however, does not meet the requirements of Article 33(3) PCT. A *vrn1* gene as claimed in claim 1 would only be inventive if the existence of such a gene would have not been known at the filing date of the present application. This does, however, not seem to be the case. D1 discloses the existence of an Arabidopsis *vrn1* gene. Therefore, an inventive step for the subject-matter of claim 1 cannot be acknowledged.

Re Item VIII

Certain observations on the international application

1. The intended function of a nucleic acid sequence, i.e. capable of specifically altering the vernalisation response of a plant, is a non-distinctive characteristic and would not render the subject-matter of claim 1 novel over the prior art (see also PCT Guidelines IV-7.6).

2. Expressions such as "derivative" and the like in present claim 5 are unclear since any DNA or amino acid sequence can be considered a "derivative" of any other sequence given enough substitutions, insertions and deletions. Limitation to a function does not resolve this problem (see above).

3. Nucleic acid sequences that only show 50% or 60% identity to SEQ ID NO:11 (Figure 7) as in present claim 2 are not supposed to code for a VRN1 polypeptide. The application does not provide sufficient support to allow such a claim (Article 5 PCT).
   Based on the cloning and sequencing *vrn1* gene it appears to be unjustified to extend the scope of the claims to structurally unrelated genes equally involved in vernalisation. The description does also not disclose such genes in a manner sufficiently clear and complete that a man skilled in the art could arrive in obtaining them (Article 5 PCT).

4. Moreover, it appears doubtful whether such a protein would solve the technical problem, namely the provision of a protein that is involved in vernalisation.

CLAIMS

1 An isolated nucleic acid molecule which comprises a VRN1 nucleotide sequence encoding a polypeptide which is capable of specifically altering the vernalisation response of a plant into which the nucleic acid is introduced and expressed.

2 A nucleic acid as claimed in claim 1 wherein the VRN1 nucleotide sequence:
(i) encodes the VRN1 polypeptide of Fig 7, or
(ii) encodes a variant polypeptide which is a homologous variant of the polypeptide shown in Fig7 and which shares at least 50%, 60%, 70%, 80% or 90% identity therewith,

3 A nucleic acid as claimed in claim 1 or claim 2 wherein the VRN1 nucleotide sequence is that shown in Fig 7 from nucleotides 269-1295 inclusive, or a sequence which is degeneratively equivalent thereto.

4 A nucleic acid as claimed in claim 1 or claim 2 wherein the VRN1 nucleotide sequence is shown in Annex I.

5 A nucleic acid as claimed in claim 1 or claim 2 wherein the VRN1 nucleotide sequence encodes a derivative of the polypeptide shown in Fig 7 by way of addition, insertion, deletion or substitution of one or more amino acids.

6 A nucleic acid as claimed in claim 1 or claim 2 wherein the VRN1 nucleotide sequence consists of an allelic or other homologous variant of the nucleotide sequence of claim 3.

7 A nucleic acid as claimed in claim 6 wherein the VRN1 nucleotide sequence is the *VRN1* paralogue *RTV1* of Figure 9.

8 An isolated nucleic acid which comprises a nucleotide sequence which is the complement of the *VRN1* nucleotide sequence of any one of the preceding claims.

9 An isolated nucleic acid for use as a probe or primer, said nucleic acid having a distinctive sequence of at least 16-24 nucleotides in length, which sequence is present in Annex I or a sequence which is degeneratively equivalent thereto, or the complement of either.

10 A nucleic acid as claimed in claim 9 which is selected from the oligonucleotides (shown below in the 5' to 3' orientation):

S63	CAACGGTTAGCCCAAAC
S64	GTTTGGGCTAACCGTTG
V11	GAGACCAGTTTTGTTTTCC
S62	GACAAATATAGGTGGAAAGG
S66	AAAGGGGAGTAGGTGGG
V7	CTCTCTGGTCTTCTCTTC
V10	GAAGAGAAGACCAGAGAG
V6	TTTTCTCATCCACTATCC
S51	TTTCTTGGATAGTGGATGAG
S65	AAAACAGGGAAGAGTAAGAAG
S52	CATTGGTTGTGTTTGGTGGG
V5	GGTCTCTATGTATTGTGC
V4	GCACAATACATAGAGACC
V12	AGATTGATTACACGACTCC
V8	CCCAGATAAGTTTGTGAG
V3	ATTCCGCTCACAACCAC
V15	GTTTGAAGTGGTTGTGAG
V14	TACCCATCACCACTTCC
S60	CAGAAGAAGGAAAGATGACC
S61	GAAGAAAGAGAGAGAGCC
V13	ACCCTTTCTTCAGAGTG

22 A transgenic plant which is obtainable by the method of claim 21, or which is a clone, or selfed or hybrid progeny or other descendant of said transgenic plant, which in each case includes a heterologous nucleic acid of any one of claims 1 to 8.

23 A plant as claimed in claim 22 which is selected from the list consisting of: rice; maize; wheat; barley; oats; rye; oil seed rape; sugar beet; maize; sunflower; soybean; sorghum; lettuce; endive; cabbage; broccoli; cauliflower; carnations; geraniums.

24 A part of propagule from a plant as claimed in claim 22 or claim 23, which in either case includes a heterologous nucleic acid of any one of claims 1 to 8.

25 An isolated polypeptide which is encoded by the VRN1 nucleotide sequence of any one of claims 1 to 7.

26 A polypeptide as claimed in claim 25 which is the VRN1 polypeptide shown in Fig 4.

27 A method of making the polypeptide of claim 25 or claim 26, which method comprises the step of causing or allowing expression from a nucleic acid of any one of claims 1 to 7 in a suitable host cell.

28 A polypeptide which comprises the antigen-binding site of an antibody having specific binding affinity for the polypeptide of claim 26.

29 A method for assessing the vernalisation phenotype of a plant, the method comprising the step of determining the presence and/or identity of a VRN1 allele therein

The Plant Cell, Vol. 10, 1973–1989, December 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

REVIEW ARTICLE

The Transition to Flowering

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INTRODUCTION

The general body plan of plants is established during embryogenesis, when the undifferentiated meristematic regions of root and shoot are set aside. However, much of plant development occurs postembryonically, through the reiterative production of organ primordia at the shoot apical meristem (SAM). In most species, the SAM initially gives rise to vegetative organs such as leaves, but at some point the SAM makes the transition to reproductive development and the production of flowers.

This change in the developmental fate of primordia initiated at the SAM is controlled by environmental and endogenous signals (Bernier, 1988; McDaniel et al., 1992). However, unlike many developmental transitions in animals, the SAM of plants is not irreversibly "committed" to reproductive development once flowering commences. In some species and genotypes under certain environmental conditions, leafy shoots are formed after flowers in a phenomenon known as inflorescence reversion (see, e.g., Battey and Lyndon, 1990; Pouteau et al., 1997). This observation implies that the genes and processes involved in the transition to flowering are required to both initiate and maintain reproductive development.

Because many species must reach a certain age or size before they can flower, the vegetative meristem is thought to first pass through a "juvenile" phase in which it is incompetent to respond to internal or external signals that would trigger flowering in an "adult" meristem. The acquisition of reproductive competence is often marked by changes in the morphology or physiology of vegetative structures—leaf shape offers one example—in a process known as vegetative phase change (Poethig, 1990; Lawson and Poethig, 1995). It is likely that some of the genes identified as important in controlling the transition from vegetative to reproductive development are also involved in vegetative phase change.

In some species, the timing of flowering is primarily influenced by environmental factors, which serve to communicate the time of year and/or growth conditions favorable for sexual reproduction and seed maturation. These factors include photoperiod (i.e., day length), light quality (spectral composition), light quantity (photon flux density), vernalization (exposure to a long period of cold), and nutrient and water availability. Other species are less sensitive to environmental variables and appear to flower in response to internal cues such as plant size or number of vegetative nodes. Flowering can also be induced by stresses such as nutrient deficiency, drought, and overcrowding. This response enables the plant to produce seeds, which are much more likely to survive the stress than is the plant itself.

Over the years, physiological studies have led to three models for the control of flowering time (reviewed in Bernier, 1988; Thomas and Vince-Prue, 1997). The florigen concept (reviewed in Lang, 1952; Evans, 1971) was based on the transmissibility of substances or signals across grafts between reproductive "donor" shoots and vegetative "recipients." It was proposed that florigen, a flower-promoting hormone, was produced in leaves under favorable photoperiods and transported to the shoot apex in the phloem. The identification of a graft-transmissible floral inhibitor also led to the concept of a competing "antflorigen." Many research years were consumed hunting for florigen in the phloem sap, but its chemical nature has remained elusive.

The inability to separate the hypothetical flowering hormones from assimilates led to a second model, the nutrient diversion hypothesis. This model proposed that inductive treatments result in an increase in the amount of assimilates moving to the apical meristem, which in turn induces flowering (reviewed in Sachs and Hackett, 1983; Bernier, 1988).

The view that assimilates are the only important component in directing the transition to flowering was superseded by the multifactorial control model, which proposed that a number of promoters and inhibitors, including phytohormones and assimilates, are involved in controlling the developmental transition (Bernier, 1988). According to this model, flowering can only occur when the limiting factors are present at the apex in the appropriate concentrations and at the right times. This model attempted to account for the diversity of flowering responses by proposing that different factors could be limiting for flowering in different genetic backgrounds and/or under particular environmental conditions.

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Genetic analysis of flowering time in pea, cereals, and Arabidopsis supports the hypothesis that the transition to flowering is under multifactorial control (reviewed in Snape et al., 1996; Weller et al., 1997; Koornneef et al., 1998b). Indeed, multiple genes that control flowering time have been identified in all three of these species. Moreover, some of these genes act to promote flowering and others to repress it; some interact with environmental variables and others appear to act autonomously.

The most striking recent advances in our understanding of the genetic control of the timing of flowering have come from work on Arabidopsis. This area of research has been extensively reviewed (see Martinez-Zapater et al., 1994; Haughn et al., 1995; Weigel, 1995; Amasino, 1996; Aukerman and Amasino, 1996; Dennis et al., 1996; Hicks et al., 1996; Madueño et al., 1996; Peeters and Koornneef, 1996; Wilson and Dean, 1996; Coupland, 1997; Koornneef et al., 1998b; Levy and Dean, 1998; Piñeiro and Coupland, 1998), and a number of key findings have emerged. Flowering involves the sequential action of two groups of genes: those that switch the fate of the meristem from vegetative to floral (floral meristem identity genes), and those that direct the formation of the various flower parts (organ identity genes). Therefore, genes that control flowering time can be expected to interact with floral meristem identity genes, which in Arabidopsis include *LEAFY* (*LFY*), *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*), *AP2*, and *UNUSUAL FLORAL ORGANS* (*UFO*). The floral meristem identity genes are themselves capable of influencing flowering time. For example, overexpression of *LFY* and *AP1* causes early formation of determinate floral meristems (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995), whereas mutations in *TFL1* affect both flowering time and meristem identity (Shannon and Meeks-Wagner, 1991). The regulation of floral meristem identity genes is under intense investigation. However, because of space constraints, this topic is covered here only briefly (for recent reviews, see Ma, 1997; Piñeiro and Coupland, 1998).

To complement earlier reviews, we describe here the current view of the control of flowering time and discuss the classic physiological studies in the context of recent molecular genetic advances. We begin by introducing the genes and mutations identified in Arabidopsis that are known to influence the timing of flowering. On the bases of their phenotypes under different growth conditions and genetic epistasis experiments, these mutants and genes are grouped into separate pathways that either promote or repress flowering. The role of DNA methylation in flowering is covered in two places to discuss separately its possible role in repression of flowering and its hypothesized role in vernalization.

In the second section, we examine the role of substances such as phytohormones that classically have been implicated in the control of flowering time and attempt to place these substances in the promotive and repressive genetic pathways. In the final section, we discuss recent data on genetic interactions that control the floral transition, and we present an updated model that attempts to summarize some of the known interactions.

GENETIC CONTROL OF FLOWERING

Arabidopsis is a facultative long-day plant; thus, long-day photoperiods are inductive, and short-day photoperiods are noninductive. The majority of Arabidopsis ecotypes are winter annuals, that is, they flower late unless they have experienced a vernalization period. This feature allows them to overwinter vegetatively and to delay flowering until favorable conditions arrive in the spring. Genes that affect flowering time in Arabidopsis have been identified through analyses of natural variation in different ecotypes and through characterization of induced mutations. The currently identified genes that are considered to play a role in flowering-time control are summarized in Figure 1 and Table 1.

Most of the genes identified by mutagenesis are derived from three rapid-cycling progenitor ecotypes: Landsberg *erecta* (*Ler*), Wassilewskija (WS), and Columbia (Col). The analysis of flowering-time variation in the naturally late-flowering ecotypes therefore complements the mutagenic approach, particularly regarding repressors of the floral transition. A number of genes—*FRI*, *FLC*, *FKR*, *JUV*, and *KRY*—and quantitative trait loci (QTLs) that are not represented in the mutant collections have been identified by this approach (Figure 1 and Table 1; reviewed in Koornneef et al., 1998b). Taken together, there are currently ~80 loci in Arabidopsis that are known to affect flowering time.

The response of flowering-time mutants to environmental treatments, such as vernalization and photoperiod (Table 1), combined with genetic analyses of epistasis, have established the existence of at least four pathways that control flowering time in Arabidopsis (Figure 2). Two of these pathways appear to monitor the endogenous developmental state of the plant. The floral repression pathway(s) may be a built-in mechanism that prevents flowering until the plant has reached a certain age or size, whereas the autonomous promotion pathway is believed to increasingly antagonize this repression as the plant develops. The other two pathways mediate signals from the environment: the photoperiodic promotion pathway is responsible for floral induction in response to inductive photoperiods, and the vernalization promotion pathway allows flowering to occur after experiencing an extended period of cold temperature (Figure 2).

Floral Repression Pathways

The identification of loss-of-function mutations that accelerate flowering in rapid-cycling ecotypes such as *Ler* reveals that even in early-flowering ecotypes, some genes act to repress flowering. Most early-flowering mutants have been categorized by their response to photoperiod (Table 1): some (e.g.,

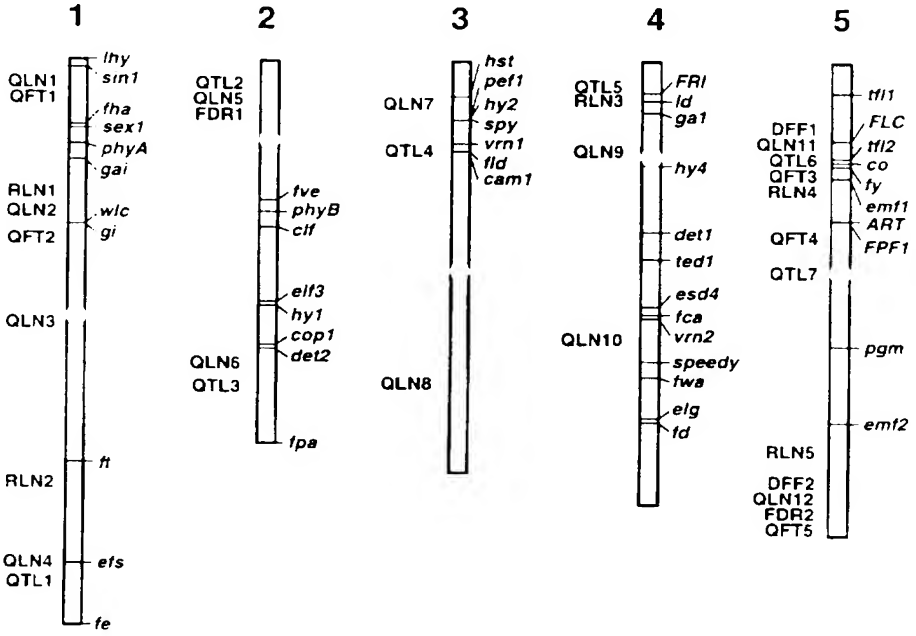

Figure 1. Genetic Map Showing the Approximate Positions of the Genes and Quantitative Trait Loci That Affect Flowering Time in Arabidopsis.

This map, which has been updated from that shown in Koornneef et al. (1998b), shows the five chromosomes as vertical bars, with the centromeres indicated by gray ellipses. Mutant loci are given in lowercase, whereas loci identified in natural populations are given in uppercase. The QTLs were initially described in the following publications: QLN1-12, Jansen et al. (1995); QFT1-5 and QTL1-7, Koornneef et al. (1998b), FDR1-2, Mitchell-Olds (1996); RLN1-5, Clarke et al. (1995); and DFF1-2, Kowalski et al. (1994).

clf, *elf1*, *elf2*, *elg*, *esd4*, *pef1*, *pef2*, *pef3*, *phyB*, *speedy*, *tfl1*, *tfl2*, and *wlc*) retain a response to photoperiod, whereas others (*elf3*, *emf1*, *emf2*, and *pif*) do not. Because this division is not absolute, the early-flowering mutants are considered here collectively, and the products of the corresponding wild-type genes are thought to act in repression of flowering.

The *EMF* genes have been considered to play a major role in repression of flowering because *emf1* and *emf2* mutants flower with essentially no preceding vegetative phase (Sung et al., 1992; Yang et al., 1995). The *EMF* genes may mediate the repression of flowering via their interactions with certain floral meristem identity genes (Figure 2). For example, *AP1* and *AG* are expressed very early in germinating *emf* seedlings, and constitutive expression of *LFY* enhances the phenotype of weak *emf1* alleles. These observations suggest that the *EMF* genes and *AP1* and *AG1* reciprocally regulate each other in a negative fashion (Figure 2; Chen et al., 1997).

Some gene products that promote flowering may act, in part, by directly or indirectly repressing *EMF* function. For example, *emf1* and *emf2* are, respectively, epistatic to *gi* and *co* (two late-flowering mutants in the photoperiodic promotion pathway; Figure 2) (Yang et al., 1995). However, when the *emf* mutations are combined with *fca* and other mutations that result in late flowering, the double-mutant plants flower after they have produced an intermediate number of leaves (Haung and Yang, 1998), which suggests that the corresponding wild-type products of these genes do not act by repressing *EMF* function.

TFL1, another floral repressor (Table 2), was cloned recently on the basis of its similarity to its Antirrhinum ortholog *CENTRORADIALIS* (*CEN*) (Bradley et al., 1997) and by T-DNA tagging (Ohshima et al., 1997). The *tfl1* mutant flowers early, and the normally indeterminate shoot apex terminates with a flower. Ordinarily, therefore, *TFL1* must function to suppress flower formation at the apex and to delay the transition from vegetative to reproductive development. Consistent with this role, overexpression of *TFL1* greatly extends the vegetative and inflorescence growth phases (Ratcliffe et al., 1998). It is likely that *TFL1* exerts this delay in flowering by repressing the function of genes such as *FCA*, *FVE*, and *FPA*, which operate in the autonomous promotion pathway (Figure 2). This is because the late-flowering phenotype conferred by mutations in these genes is epistatic to *tfl1* (Ruiz-Garcia et al., 1997; T. Page and C. Dean, unpublished results).

CLF and *WLC* (Table 2) act to delay flowering by repressing certain floral meristem identity genes. The *clf* mutant expresses *AG* ectopically in leaves, inflorescence stems, and flowers (Goodrich et al., 1997), and *wlc* expresses *AG* and

Table 1. Genes and Mutations That Affect Flowering Time in Arabidopsis[a]

Locus		Description[b]	Environ Response[c] Ppd	Vern	References
ADG1	*ADP GLUCOSE PYROPHOSPHORYLASE1*	Mutants lack leaf starch and flower late, primarily in SDs	+	ND	Lin et al. (1988)
ART	*AERIAL ROSETTE*	In combination with another locus, probably *FRI*, delays flowering of the axillary meristems, giving rise to aerial rosettes in LDs	+	+	Grbic and Bleecker (1996)
CAM1	*CARBOHYDRATE ACCUMULATION MUTANT1*	Mutants flower late and have increased starch in leaves	–		Eimert et al. (1995)
CCA1	*CIRCADIAN CLOCK ASSOCIATED1*	Overexpression results in long hypocotyls, abolished circadian rhythms, and late flowering		ND	Wang and Tobin (1998)
CLF	*CURLY LEAF*	Mutants flower early, have upwardly curled leaves, and express *AGAMOUS* ectopically	–	ND	Goodrich et al. (1997)
CO (=*FG*)	*CONSTANS*	Mutants flower late		–	Redei (1962)
COP1 (=*FUS1*)	*CONSTITUTIVE PHOTOMORPHOGENIC1*	Mutants flower early in SDs and are constitutively photomorphogenic when germinated in the dark	–	ND	Deng et al. (1991)
DET1 (=*FUS2*)	*DEETIOLATED1*	Mutants have a phenotype similar to *cop1*	–	ND	Chory et al. (1989b)
DET2	*DEETIOLATED2*	Mutants flower late and exhibit pleiotropic defects in dark- and light-grown development	ND	ND	Chory et al. (1991)
ELF1, 2	*EARLY FLOWERING1* and *2*	Mutants flower early	+	ND	Zagotta et al. (1992)
ELF3	*EARLY FLOWERING3*	Mutants flower early in SDs, and have a long hypocotyl primarily in B and no circadian rhythm in cL		ND	Zagotta et al. (1992)
ELG	*ELONGATED*	Mutants flower early and have long hypocotyls	+	ND	Halliday et al. (1996)
EMF1,2	*EMBRYONIC FLOWER1* and *2*	Mutants flower extremely early and have severe pleiotropic effects on leaf and flower morphology	–	ND	Sung et al. (1992)
ESD4	*EARLY IN SHORT DAYS4*	Mutants flower early, have club-shaped siliques, and form a terminal flower	±	ND	Coupland (1995)
FCA		Mutants flower late and are strongly responsive to vernalization	+	+	Koornneef et al. (1991)
FD		Mutants flower late	+	–	Koornneef et al. (1991)
FE		Mutants flower late	±	±	Koornneef et al. (1991)
FHA (=*CRY2*)	(=*CRYPTOCHROME2*)	Mutants flower mildly late and have a long hypocotyl in low intensity B	–	+	Koornneef et al. (1991)
FKR	*FLOWERING KIRUNA*	Recessive alleles cause late flowering			J.E. Burn et al. (1993)
FLC	*FLOWERING LOCUS C*	Dominant alleles such as *FLC*-Col enhance the effect of late alleles at *FRI* and *LD* and of mutations at *fca*, *fpa*, and *fve*, in the *Ler* background	+	+	Koornneef et al. (1994), Lee et al. (1994b)
FLD	*FLOWERING LOCUS D*	Dominant alleles cause late flowering, which requires a late allele of *FLC* for full effect	+	+	Sanda and Amasino (1996)
FPF1	*FLOWERING PROMOTING FACTOR1*	Overexpression causes early flowering in LDs and SDs	+	ND	Kania et al. (1997)
FPA		Mutants flower late	+	+	Koornneef et al. (1991)
FRI (–*FLA*)	*FRIGIDA*	Dominant alleles cause late flowering, which is suppressed by vernalization	+	+	Napp-Zinn (1957)
FT		Mutants flower late	±	±	Koornneef et al. (1991)
FVE		Mutants flower late	+	+	Koornneef et al. (1991)
FWA (–*FTS*)		Mutants flower late	+	+	Koornneef et al. (1991)
FY		Mutants flower late	+	+	Koornneef et al. (1991)
GA1		Mutants flower late in LDs and do not flower in SDs	–	+	Koornneef and van der Veen (1980)
GAI	*GIBBERELLIN INSENSITIVE*	Mutants flower late in SDs	+	–	Koornneef et al. (1985)
GI (–*FB*)	*GIGANTEA*	Mutants flower late and have increased starch in leaves	–	–	Redei (1962)

(continued)

Table 1. Continued

Locus		Description[b]	Environ Response[c] Ppd	Vern.	References
HST	*HASTY*	Mutants have a shortened juvenile vegetative phase and flower early	−	ND	Telfer and Poethig (1998)
HY1,2	*LONG HYPOCOYTL1* and *2*	Mutants flower early and have pale-green young rosettes and long hypocotyls	+	ND	Koornneef et al. (1980); Chory et al. (1989a)
HY4 (=*CRY1*)	*LONG HYPOCOTYL4* (=*CRYPTOCHROME1*)	Mutants have long hypocotyls in B and flower late in certain ecotypic backgrounds	+	ND	Koornneef et al. (1980)
JUV	*JUVENALIS*	Recessive alleles cause late flowering, which is suppressed by vernalization	+	+	Napp-Zinn (1957)
KRY	*KRYOPHILA*	Recessive alleles cause late flowering, which is suppressed by vernalization	+	+	Napp-Zinn (1957)
LD	*LUMINIDEPENDENS*	Mutants flower late in combination with a late allele of *FLC*	+	+	Rédei (1962)
LHY	*LATE ELONGATED HYPOCOTYL*	Overexpression results in long hypocotyls, abolished circadian rhythms, and late flowering	−	-	Schaffer et al. (1998)
PEF1	*PHYTOCHROME-SIGNALING EARLY-FLOWERING*	Mutants flower early and are similar to *hy1* and *hy2*	+	ND	Ahmad and Cashmore (1996)
PEF2, 3	*PHYTOCHROME-SIGNALING EARLY-FLOWERING2* and *3*	Mutants flower early and are similar to *phyB*	+	ND	Ahmad and Cashmore (1996)
PGM	*PHOSPHOGLUCOMUTASE*	Mutants lack starch and flower late, primarily in SDs	+	+	Caspar et al. (1985)
PHYA (=*HY8, FHY2*)	*PHYTOCHROME A* (=*LONG HYPOCOTYL8*)	Mutants have long hypocotyls in far-red light and are impaired in day-length perception	−	ND	Whitelam et al. (1993)
PHYB (=*HY3*)	*PHYTOCHROME B* (=*LONG HYPOCOTYL3*)	Mutants flower early, are pale green, and have long hypocotyls and petioles	+	ND	Koornneef et al. (1980)
PIF	*PHOTOPERIOD INSENSITIVE*	Mutants flower early, have small curled leaves, and are dwarfed	−	ND	Hicks et al. (1996)
SEX1	*STARCH EXCESS1*	Mutants have increased starch in leaves and flower late (except in cL)	+	+	Caspar et al. (1991)
SIN1	*SHORT INTEGUMENT1*	Mutants flower late and are female sterile	+	−	Ray et al. (1996)
SPEEDY (=*EBS*)	(=*EARLY BOLTING IN SHORT DAYS*)	Mutants flower early	±	ND	Koornneef et al. (1998b)
SPY	*SPINDLY*	Mutants flower early and resemble plants treated with gibberellins	ND	ND	Jacobsen and Olszewski (1993)
TED1		Mutants suppress *det1* and flower late	+	ND	Pepper and Chory (1997)
TFL1	*TERMINAL FLOWER1*	Mutants flower early and have determinate shoot growth and replacement of coflorescences with flowers	+	ND	Shannon and Meeks-Wagner (1991)
TFL2	*TERMINAL FLOWER2*	Mutants are similar to *tfl1* but flower even earlier and are markedly reduced in size	±	ND	Hicks et al. (1996)
VRN1	*VERNALIZATION1*	Mutants flower late only after vernalization	+	−	Chandler et al. (1996)
VRN2	*VERNALIZATION2*	Mutants flower moderately late in combination with *fca* and have a reduced vernalization response	+	−	Chandler et al. (1996)
WLC	*WAVY LEAVES AND COTYLEDONS*	Mutants flower early, have reduced size, and display a charactenstic waving and rolling of the leaves	±	ND	Bancroft et al. (1993)

[a] For up-to-date information on the cloning of genes involved in flowering time, refer to "The Flowering Web" (http://www.salk.edu/LABS/pbio-w/flower_web.html).

[b] B, blue light; cL, continuous light; LD, long day; SD, short day.

[c] Environmental response of the mutant or otherwise indicated allele to flower earlier under inductive photoperiods (Ppd.) and after vernalization (Vern.). (+), strongly sensitive; (±), weakly sensitive; (−), insensitive; ND, not determined.

AP3 ectopically in leaves. Thus, the wild-type function of *CLF* and *WLC* is to prevent the expression of the floral meristem identity genes in vegetative tissue. The *CLF* gene shares sequence homology with the Drosophila polycomb group of genes, which are involved in maintaining the repression of homeotic genes (Goodrich et al., 1997). The *wlc* mutant displays hypomethylation of repetitive sequences associated with the centromeres (C. Hutchison and C. Dean, unpublished results); thus, reduced methylation may directly alleviate the repression of *AG* and *AP3* expression in leaves. Similarly, induced hypomethylation resulting from constitutive expression of an antisense methyltransferase gene resulted in ectopic expression of *AG* and *AP3* and early flowering (Finnegan, 1996). Thus, methylation may play an important role in the repression of the floral transition.

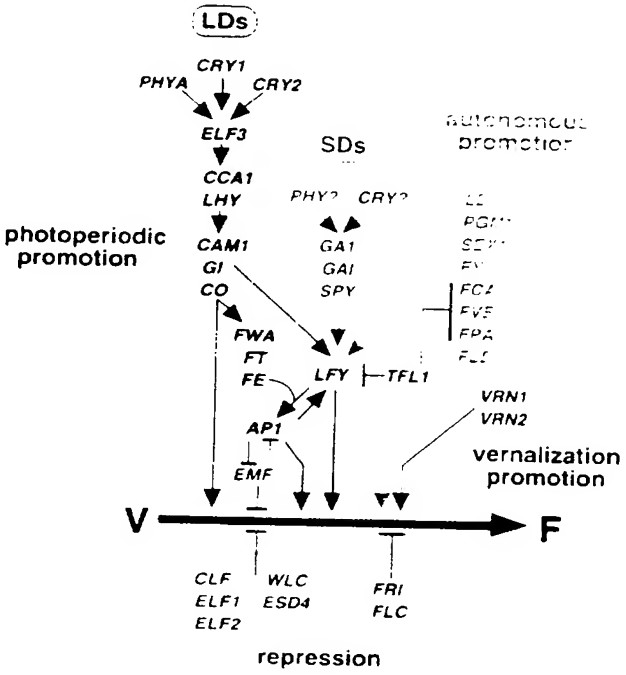

Figure 2. Genetic Pathways That Control Flowering Time in Arabidopsis and Proposed Interactions among Some of the Genes Involved.

The horizontal line symbolizes the vegetative (V) to floral (F) transition, with the promotive and repressive pathways exerting their influence on this switch. Four pathways are shown: repression (green), autonomous promotion (red), photoperiodic promotion under long days (LDs; dark blue) and short days (SDs; light blue), and vernalization promotion (pink). Genes that influence both floral meristem identity and flowering time are shown in black. Promotive (arrows) and repressive (T-bars) interactions are based on genetic epistasis experiments and analysis of gene expression in mutant and overexpressing lines. Not all interactions have been tested directly, and little is known about how the floral repressors interact with the various promotive pathways; thus, most of the repressors have simply been represented below the horizontal line. Therefore, this model, which is an updated combination of those published by Koornneef et al. (1998b) and Nilsson et al. (1998), does not fully represent the complexity of the interactions between genes and pathways that control flowering time in Arabidopsis.

Methylation appears to play a role in the regulation of flowering time by the *FWA* gene. Working with the *ddm1* mutant, which has decreased DNA methylation but unaltered methyltransferase activity (Richards, 1997), Kakutani et al. (1996) noted late flowering as a frequently appearing phenotype in repeatedly self-pollinated *ddm1* lines. *FTS*, the dominant locus conferring this late-flowering phenotype, was mapped genetically (Kakutani, 1997) and localized close to *FWA*, which was previously characterized by Koornneef et al. (1991) as a dominant mutation conferring late flowering. Subsequent analysis of the methylation status of the genomic region surrounding the *FWA* locus in *ddm1* and in EMS-induced *fwa* alleles showed the region to be hypomethylated (Koornneef et al., 1998b). Therefore, the wild-type product of the *FWA* gene may encode a repressor of flowering that normally is downregulated by methylation. However, because there is precedence for local hypermethylated sites within a hypomethylated region of a gene (see, e.g., Jacobsen and Meyerowitz, 1997), it is difficult to predict whether or not *FWA* expression will be up- or downregulated in the *fwa* mutant. Ronemus et al. (1996) speculated that a general and gradual increase in methylation during development could serve to change meristem competency and determinacy as a plant ages. It will interesting to test whether such a gradient of methylation exists in Arabidopsis and whether alleviation of the autonomous repression of flowering depends, at least in part, on changes in methylation at specific loci such as *FWA*.

Analysis of the natural variation in flowering time has revealed that the early-flowering ecotypes such as Ler and Col can themselves be considered as mutants in genes conferring strong repression of the floral transition. Crosses between a number of winter and spring Arabidopsis ecotypes revealed that late flowering and a requirement for vernalization segregated as a dominant monogenic trait (Sanda et al., 1997) that mapped to the *FRI* locus (J.E. Burn et al., 1993; Lee et al., 1993; Clarke and Dean, 1994). The recent map-based cloning of *FRI* has revealed that Ler and Col are likely to carry loss-of-function *FRI* alleles (U. Johanson and C. Dean, unpublished data).

Dominant alleles at a second locus, *FLC*, are required for the full repression of flowering by *FRI* (Lee et al., 1994b; Aukerman and Amasino, 1996). Most ecotypes carry dominant alleles at *FLC*, but Ler and the C24 ecotype carry recessive alleles (Michaels and Amasino, 1995). Map-based cloning of *FLC* is nearing completion (S.D. Michaels and R.M. Amasino, personal communication), and therefore, the basis of this variation can soon be analyzed at the molecular level. Future studies will also be able to address how the

Table 2. Cloned Arabidopsis Genes That Affect Flowering Time[a]

Gene	Sequence Similarity and Probable Function
Promoters of flowering	
ADG-1	ADP glucose pyrophosphorylase, involved in starch metabolism
CO	Putative transcription factor with two zinc fingers
DET2	Steroid 5α-reductase, an enzyme involved in brassinolide biosynthesis
FCA	RNA binding protein with a protein–protein interaction domain
FHA	Cryptochrome 2, a flavin-containing blue light photoreceptor
FPF-1	Novel protein that may be involved in signaling or response to GAs
FT	*TFL1* homolog[b]
GA1	*ent*-kaurene synthetase A, an enzyme involved in GA biosynthesis
GAI	Member of a novel family of putative transcription factors
GI	Novel protein with putative membrane-spanning regions[c]
LD	Glutamine-rich homeobox transcription factor
PGM	Phosphoglucomutase, involved in starch metabolism
PHYA	Light-labile R-FR light photoreceptor
Repressors of flowering	
CCA1	MYB-related transcription factor; *LHY* homolog
CLF	Homology to *Enhancer of Zeste*, a Drosophila polycomb-group gene
ELF3	Novel protein[d]
ESD4	Novel protein
LHY	MYB-related transcription factor; *CCA1* homolog
PHYB	Light-stable R-FR light photoreceptor
SPY	*O*-linked *N*-acetylglucosamine transferase, involved in modification of proteins
TFL1	Similarity with phosphatidylethanolamine binding proteins
WLC	Novel protein[e]

[a] For up-to-date information on the cloning of genes involved in flowering time, refer to "The Flowering Web" (http://www.salk.edu/LABS/pbio-w/flower_web.html).
[b] T. Araki and D. Weigel, personal communication.
[c] K. Lee, G. Coupland, S. Fowler, and J. Putterill, personal communication.
[d] D.R. Meeks-Wagner, personal communication.
[e] C. Hutchison and C. Dean, unpublished data.

vernalization promotion pathway (see below) is able to bypass the repression of flowering mediated by *FRI* and *FLC* (Figure 2).

Given that so many genes are involved in the regulation of flowering time in Arabidopsis, it is interesting that a major determinant of both the natural variation in flowering time and the requirement for vernalization is allelic variation at *FRI*. *FRI* maps close to one of the two major QTLs that confer a vernalization requirement in *Brassica* spp (Osborn et al., 1997). Thus, an important question to address in the future is whether *FRI* orthologs correspond to flowering-time loci in a number of plant species.

Autonomous Promotion Pathway

The identification of loss-of-function mutations that delay flowering of rapid-cycling ecotypes reveals genes that act to promote flowering. Many of these late-flowering mutants have been categorized by their response to vernalization and photoperiod and in epistasis experiments (Table 1; Koornneef et al., 1991, 1998a). One group of mutants (*co*, *fd*, *fe*, *fha*, *ft*, *fwa*, and *gi*) show little response to photoperiod or vernalization, and the corresponding genes are thought to act in the photoperiodic promotion pathway (Figure 2). A second group of mutants (*fca*, *fpa*, *ld*, *fve*, and *fy*) respond strongly to vernalization but flower even later under noninductive photoperiods. Because the products of the corresponding wild-type genes appear to promote flowering independently of photoperiod, these genes are considered to act in the autonomous promotion pathway (Figure 2). Moreover, the fact that these mutants respond to vernalization suggests that the vernalization promotive pathway acts redundantly with the autonomous promotion pathway in these early-flowering ecotypes.

Two genes of the autonomous promotion pathway encode proteins whose function may be to regulate the expression of other genes (Table 2). *LD* encodes a putative homeodomain protein, and although the *LD* transcript is expressed throughout the plant, it is most abundant in the shoot and root apices (Lee et al., 1994a; Aukerman and Amasino, 1996). *FCA* encodes a protein with RNA binding and protein–protein interaction domains (Macknight et al., 1997). The RNA binding domains of FCA are similar to those

of the Drosophila proteins SX-1 and ELAV, which regulate alternative splicing of pre-mRNA transcripts important for sex determination and neuronal differentiation (Macknight et al., 1997). The *FCA* transcript is itself alternatively spliced, and increasing the levels of specific *FCA* transcripts results in earlier flowering (R. Macknight and C. Dean, unpublished results).

Analysis of the interaction of *FCA* with meristem identity genes indicates that *FCA* function is required for both activation and competence to respond to *LFY* and *AP1* (T. Page and C. Dean, unpublished results). FCA, or downstream gene products, appear to act in a cell non-autonomous manner, because even in plants in which a large proportion of the two inner layers of the SAM (i.e., L2 and L3) are genotypically *fca*, bolting and flowering are normal (Furner et al., 1996).

Transmissible signals that promote flowering are also the focus of recent work by Colasanti et al. (1998). The maize *id1* mutation confers late flowering and altered floral development. *ID1* encodes a protein with zinc finger motifs, suggesting that it acts as a transcriptional regulator. Several observations led Colasanti et al. (1998) to propose that *ID1* may be involved in the production or transport of a transmissible signal. For example, *id1* plants do not flower under field conditions, and plants containing an increasing proportion of transposon-induced wild-type *ID1* sectors in a mutant *id1* background flower progressively earlier (Colasanti et al., 1998). Taken together, these experiments suggest that ID1 is required to produce and/or modulate the activity of a signal that originates in immature leaves and influences reproductive development in the SAM.

That leaves are required to determine the developmental potential of the apex has also been established using cultured maize apices. Excised apices revert to producing a full set of leaves before they produce flowers, irrespective of how many leaves had been produced before they were placed in culture (Irish and Jegla, 1997). However, leaving the four to six youngest leaf primordia on the excised apices prevents the resetting of the developmental program, indicating that some signal from the leaves influences development of the apex.

Photoperiodic Promotion Pathway

Plants detect light in at least five regions of the visible spectrum by using at least three classes of photoreceptors. Blue light and ultraviolet-A are detected by the cryptochromes, red (R) and far-red (FR) light are detected by the phytochromes, and ultraviolet-B is detected by an as-yet-unidentified photoreceptor (Thomas and Vince-Prue, 1997). In Arabidopsis, there are at least five phytochromes (PHYA to PHYE) and two cryptochromes (CRY1 and CRY2) (Thomas and Vince-Prue, 1997). These photoreceptors typically have been characterized by the effect they have on seedling morphogenesis under different light conditions. Several Arabidopsis mutants that were originally isolated based on abnormal seedling photomorphogenesis are also affected in flowering time. These include *cop1*, *det1*, *det2*, *hy1*, *hy2*, *hy4*, *phyA*, *phyB*, *pef1*, *pef2*, and *pef3* (Table 1). Conversely, several mutants isolated based on their flowering-time phenotypes were subsequently found to exhibit abnormal seedling photomorphogenesis. These include *elf3*, *elg*, *fha*, and *lhy* (Table 1).

The role of photoperiod in flowering was conclusively demonstrated by Garner and Allard in the 1920s in their classic experiments with the Maryland Mammoth mutant of tobacco and the Biloxi variety of soybean (reviewed in Thomas and Vince-Prue, 1997). Recent genetic studies have begun to identify molecular components of the photoperiodic promotion pathway (Figure 2), and an overall picture of how Arabidopsis perceives and responds to inductive photoperiods is beginning to emerge.

The pathway begins with photoreceptors (such as PHYA and CRY2), which initiate signals that interact with a circadian clock and entrain the circadian rhythm. Somehow, day length is measured, and when the length of the dark period decreases below a critical length, genes that promote flowering (such as *CO*) are activated. This activation leads, in turn, to the upregulation of floral meristem identity genes and, thereafter, flowering.

In Arabidopsis, light quality affects flowering time, with R light inhibiting and FR light promoting flowering (Martinez-Zapater et al., 1994). The phenotype of *phyB* mutants (Table 1) suggests that PHYB normally plays a role in inhibiting flowering under high R to FR conditions but is not involved in day-length perception (Koornneef and Peeters, 1997). Physiological studies on multiple mutant combinations suggest that in addition to PHYB, other light-stable phytochromes also regulate flowering in response to light quality (Koornneef and Peeters, 1997). In contrast, mutations in *PHYA*, which encodes a light-labile photoreceptor, prevent perception of low-fluence-rate, FR-enriched day-length extensions that promote flowering. These observations suggest that PHYA is involved in both day-length perception and promotion of flowering by inductive photoperiods (Figure 2; Koornneef and Peeters, 1997).

Blue light alone promotes flowering in Arabidopsis, and the product of the *FHA* gene has recently been shown to encode CRY2, one of the two cryptochromes thus far identified in Arabidopsis (Guo et al., 1998). Transgenic plants overexpressing *CRY2* flowered earlier than did the wild type and had increased levels of *CO* mRNA (Guo et al., 1998), suggesting that blue light promotes flowering via CRY2 and CO (see below). Furthermore, the level of *CO* mRNA was found to be reduced in *cry2* mutants grown under long days but not under short days (Guo et al., 1998), thereby providing a possible explanation for the basis of the original *fha* late-flowering phenotype. Because the levels of both PHYA and CRY2 proteins drop rapidly and dramatically in the light (Thomas and Vince-Prue, 1997), they could fulfill the role of providing information about light/dark transitions to the circadian clock.

CRY1, the other cryptochrome in Arabidopsis, was originally identified as the *hy4* mutant, which has a long hypocotyl under blue light (Table 1). *hy4* is sensitive to photoperiod and is not delayed in flowering in a L*er* background under white light and inductive photoperiods. However, in the presence of non-L*er* alleles of *FLC* and in blue-enriched light, *hy4* is late flowering and exhibits photoperiodic sensitivity (Bagnall et al., 1996; Koornneef and Peeters, 1997). Therefore, CRY1 is involved in the promotion of flowering, but its interaction with floral promotion pathways is unclear.

Several genes that affect photoperiodic sensitivity and that may encode components of the circadian clock itself have been identified. *CCA1* and *LHY* RNA levels oscillate in a rhythmic fashion, and overexpression of either gene results in long hypocotyls and late flowering (Schaffer et al., 1998; Wang and Tobin, 1998). Constitutive expression of either *CCA1* or *LHY* also abolishes or alters the circadian expression of their own transcripts as well as several other genes, which suggests that CCA1 and LHY negatively regulate their own expression (Wang and Tobin, 1998).

Another likely component of the circadian clock is *TOC1*, which was identified as a semi-dominant mutation that shortened the period length of the circadian clock by 2 to 3 hr (Somers et al., 1998). The *toc1* mutation reduces the sensitivity of plants to photoperiod and causes early flowering under short days, indicating that quantitative changes in the pace of the circadian clock, not rhythmicity/arhythmicity alone, can alter flowering time.

ELF3 may mediate the interaction of light signals generated by the photoreceptors with the circadian clock (Figure 2). The phenotype of the *elf3* mutant (Table 1) suggests that the wild-type product of this gene is involved in repressing flowering under noninductive photoperiods. However, the conditional arhythmicity of the *elf3* mutant suggests that *ELF3*, which has recently been cloned (Table 2), does not function in the circadian clock itself (Hicks et al., 1996; Koornneef and Peeters, 1997).

The circadian clock is believed to affect the expression of downstream genes that operate in the photoperiodic promotion pathway, including *CO* (Table 2) (Putterill et al., 1995). *CO* mRNA is expressed throughout the plant and is more abundant in plants grown under long days compared with short days (Piñeiro and Coupland, 1998). *GI*, which has recently been cloned (Table 2), probably acts upstream of *CO* (Figure 2), because the phenotype of plants that overexpress *CO* is epistatic to the *gi* mutation (Piñeiro and Coupland, 1998).

Several lines of evidence suggest that the level of CO activity in L*er* plants is directly correlated with flowering time (reviewed in Piñeiro and Coupland, 1998). Using a glucocorticoid-inducible system, Simon et al. (1996) demonstrated that induction of CO activity is sufficient to rapidly cause flowering under short days and to initiate transcription of *LFY* and *TFL1* as rapidly as when these genes are induced by transfer to inductive photoperiods. However, levels of *AP1* mRNA increase more slowly after CO activation than they do in response to inductive photoperiods (Simon et al., 1996). These data suggest that CO acts in a pathway that is sufficient to activate *LFY* and *TFL1* transcription but that rapid activation of *AP1* requires an additional pathway (Figure 2). Interestingly, genetic analyses by Ruiz-Garcia et al. (1997) have placed *CO* and *TFL1* in different genetic pathways, so the rapid activation of *TFL1* transcription remains to be explained.

Vernalization Promotion Pathway

Another seasonal cue in temperate zones is a winter period, and many species require exposure of imbibed seeds or vegetative plants to a period of cold temperature (typically 2 to 8 weeks at ~4°C) in order to flower. This process, known as vernalization, is slow and quantitative but requires active metabolism (reviewed in Chouard, 1960; Vince-Prue, 1975). The site of perception of vernalization is the shoot apex (e.g., Curtis and Chang, 1930; Metzger, 1988), but all actively dividing cells, not only those at the shoot apex, may be capable of responding to vernalization (Wellensiek, 1964). Unlike photoperiodic induction, vernalization prepares the plant to flower but does not itself evoke flowering. That is, there is a clear temporal separation between cold treatment and flowering, which commonly occurs after a period of growth at warmer temperatures. Vernalization is required in each generation for winter annuals and biennials and each growth year for perennials, which suggests that meiosis or some other aspect of reproductive growth resets the requirement for vernalization.

The features of vernalization suggest that an epigenetic mechanism may be responsible for the establishment, persistence, and resetting of whatever self-perpetuating changes occur during or subsequent to exposure to cold. The observations that the flowering of late-flowering, vernalization-sensitive Arabidopsis mutants is accelerated by azacytidine treatment (J.B. Burn et al., 1993) and that cold treatment leads to specific changes in gibberellin (GA) metabolism (Hazebroek and Metzger, 1990; Hazebroek et al., 1993) led J.B. Burn et al. (1993) to propose that vernalization causes a specific reduction in cytosine methylation. This reduction, J.B. Burn et al. (1993) hypothesized, results in the activation of the gene encoding kaurenoic acid hydroxylase, an enzyme that catalyzes an early step in GA biosynthesis. Indeed, when general levels of methylation were reduced in wild-type plants by introducing a transgene expressing an antisense version of a methyltransferase gene (antisense-*MET1*), developmental abnormalities and early flowering were observed (Finnegan, 1996; Finnegan et al., 1998). However, the role of methylation in vernalization is still unclear, because substantial demethylation did not prevent vernalization from fully accelerating flowering in these lines, nor did it prevent resetting of the vernalization requirement in the progeny of antisense-*MET1* plants (Finnegan et al., 1998).

One approach to understanding the molecular basis of vernalization has been to isolate mutants of Arabidopsis that

are specifically impaired in their response to cold treatment (Chandler et al., 1996). The starting point for this genetic screen was *fca*, a late-flowering mutant whose phenotype can be completely corrected by a period of vernalization. *fca* plants were mutagenized, and a population of progeny plants were vernalized and screened for individual plants that flowered late, that is, which no longer exhibited a strong response to vernalization. Of these candidate double mutants, those that flowered no later than *fca* itself without cold treatment were selected for further characterization (Chandler et al., 1996). Such *vrn* mutants may be defective either in the perception of cold temperature or in the transduction of the cold signal by the vernalization promotion pathway (Figure 2). An initial screen identified five independent recessive *vrn* mutations in at least three complementation groups (Chandler et al., 1996), and a second screen identified five additional mutants, which have not yet been assigned to complementation groups (Y.Y. Levy and C. Dean, unpublished results). Two mutants, *vrn1* and *vrn2* (Table 1), have been characterized in some detail and are being cloned by chromosome walking. Both *vrn1* and *vrn2* have a normal acclimation response, indicating either that they are downstream of a cold-perception pathway common to acclimation and vernalization or that cold perception occurs via independent pathways in these two responses (Chandler et al., 1996). Analysis of the *VRN* genes should reveal some of the molecular components involved in promotion of flowering by vernalization.

INTEGRATING PHYSIOLOGY AND GENETICS: FLORAL SIGNALS AND GENETIC PATHWAYS

Considerable physiological analysis has led to certain compounds and processes being implicated in controlling the floral transition. These include the role of sugars, cytokinins, and GAs. In this section, we discuss the role of these substances in flowering and try to place them within the promotive and repressive pathways.

The Role of Carbohydrates in Flowering

Compelling evidence that sucrose may function in long-distance signaling during floral induction comes from studies of *Sinapis alba*, a long-day plant in the mustard family. After induction of flowering in *S. alba* by either a single long day or a displaced short day, the concentration of sucrose in the phloem reaching the apex increases rapidly and transiently (Bernier et al., 1993). Furthermore, this pulse of sucrose precedes the increase in cell division that is normally observed in the SAM upon floral induction. The sucrose reaching the apex appears to be derived from the mobilization of stored carbohydrates, most likely starch in the leaves and stems, because plants induced by a displaced short day receive the same photosynthetic input as plants maintained under noninductive photoperiods (Bernier et al., 1993).

In Arabidopsis, L*er* plants grown in darkness with their apices in contact with sucrose-containing medium flower with the same number of leaves as do plants grown under long days (Roldán et al., 1997). In contrast, sucrose has a significant effect on the flowering of vernalization-requiring ecotypes Leiden and Stockholm, which flower early when grown under these conditions and with approximately the same number of leaves as L*er* (Roldan et al., 1997). Furthermore, sucrose alone, whether supplied in the dark or in the light, is responsible for most of this acceleration. Therefore, supplying sucrose to these late-flowering ecotypes bypasses the inhibition of flowering normally conferred by the existence of dominant alleles at *FRI* and *FLC* (Table 1). Sucrose also accelerates the flowering of *fve*, *fpa*, *fca*, *co*, and *gi* but not of *ft* and *fwa* (Roldán et al., 1997). This result implies that *FVE*, *FPA*, *FCA*, *CO*, and *GI* function in processes that are either upstream of or separate from control of sucrose availability to the vegetative apex, whereas *FT* and *FWA* function in processes downstream of this control point.

Further genetic evidence connecting carbohydrate metabolism with control of flowering is available, but the nature of this connection is unclear. For example, there are at least five Arabidopsis mutants, *adg1*, *cam1*, *gi*, *pgm*, and *sex1*, which are altered in starch synthesis, accumulation, or mobilization and which flower late under some conditions (Table 1). The flowering time of *cam1* and *gi* is not influenced by photoperiod, and therefore, both are likely to act in the photoperiodic promotion pathway (Eimert et al., 1995). *pgm* and *sex1* mutants flower later in short days than they do in long days and so fall into the autonomous promotion pathway. Flowering of these mutants is accelerated by cold treatment, suggesting that vernalization does not depend on normal starch metabolism (Bernier et al., 1993).

Phytohormones

The role of GAs in the transition to flowering has been difficult to establish. On the one hand, there are many examples in which the abundance or composition of endogenous GAs changes under conditions that induce flowering (Pharis and King, 1985). Furthermore, because applying certain GAs can induce flowering in some species, there has been an emphasis on the study of GAs in floral initiation and in the search for florigen (reviewed in Chouard, 1960; Evans, 1971; Zeevaart, 1983; Thomas and Vince-Prue, 1997). On the other hand, applied GAs are rarely effective at inducing flowering in short-day plants. Moreover, they generally inhibit flowering of woody angiosperms, although they do promote flowering of conifers (Pharis and King, 1985). Even within long-day plants, the same GA can have a different effect in different species. For example, 2,2-dimethyl GA_4 has potent florigenic activity when applied to *Lolium temulentum* but has no effect on flowering in *S. alba* (Bernier et al., 1993).

In Arabidopsis, signaling mediated by GAs appears to play a promotive role in flowering, particularly under noninductive photoperiods (Figure 2). Application of GAs accelerates flowering of wild-type plants under short days (Langridge, 1957) and of the late-flowering mutants *fb*, *fca*, *fd*, *fe*, *co*, *fpa*, *ft*, *fve*, and *fwa* (Table 1) under long days (Chandler and Dean, 1994). Under noninductive photoperiods, the *ga1* mutant (Table 1) does not flower unless provided with GAs (Wilson et al., 1992), and the *gai* mutant (Table 1) flowers very late. Furthermore, *spy* (Table 1), a mutant considered to exhibit constitutive GA-mediated signal transduction, flowers early (Jacobsen and Olszewski, 1993), as do plants constitutively expressing *FPF1*, a gene that appears to be involved in GA-mediated signal transduction or responsiveness to GAs (Table 1; Kania et al., 1997).

The role of GAs in activation of the *LFY* promoter has recently been analyzed (Blázquez et al., 1998). The basal level of *LFY* promoter activity is lower in *ga1* mutants, and the upregulation by long days is delayed. In contrast, *LFY* activity is slightly higher in a *spy* mutant grown in short days, correlating with an acceleration of flowering. A cauliflower mosaic virus 35S–*LFY* transgene was also found to rescue flowering in *ga1* mutant plants in short days. Thus, GAs promote flowering in Arabidopsis at least in part by activating *LFY* expression. Blázquez et al. (1998) also analyzed the direct effect of GA_3 with and without sucrose on *LFY* promoter activity. GA_3 alone had no effect, sucrose produced a small increase, and both together had a synergistic effect. This requirement for two activation signals for maximal effect may account for observations with excised *Lolium* apices (McDaniel and Hartnett, 1996). In this study, photoperiodic induction was found to result from two signals acting at the apex. One of these signals has not been identified (but from this analysis, it is possibly sucrose), and the other is GA (McDaniel and Hartnett, 1996).

The role of GAs in vernalization has received particular attention because in some species, application of GAs to vegetatively growing plants can substitute for cold treatment (see Chouard, 1960; Lang, 1965; Evans, 1971; Zeevaart, 1983; Martínez-Zapater et al., 1994). However, in the majority of species examined, including most cereals and nonrosette plants, application of GAs is not sufficient to overcome a requirement for vernalization (Chouard, 1960; Lang, 1965; Evans, 1971; Zeevaart, 1983). Because GAs are involved in flowering processes such as floral evocation (McDaniel and Hartnett, 1996) and bolting (Metzger, 1990), which occur well after the cold treatment, it is possible that application of GAs can simply bypass vernalization completely. Consistent with this possibility is the notion that vernalization may increase the sensitivity of plants to GAs but that GAs have no direct role in the process of vernalization itself (Chouard, 1960).

Further indication that GAs may not play a role in vernalization in Arabidopsis comes from experiments with *ga1-3* (Table 1), a mutant severely impaired in GA biosynthesis (Sun and Kamiya, 1994). When combined with *fca*, which responds strongly to vernalization, the *ga1-3 fca* double mutants still exhibit a robust vernalization response (J. Chandler and C. Dean, unpublished data). However, because *ga1-3* plants still contain residual GAs (T.-p. Sun, personal communication; Zeevaart and Talón, 1992), this result must be interpreted with caution. In summary, the precise role of GAs in the transition to flowering is unclear. Potential tissue-specific changes in GA biosynthesis and sensitivity need to be addressed, as does the potential existence of as-yet-undiscovered florigenic GAs (for a discussion of this possibility, see Evans, 1971; Zeevaart, 1983).

GAs are not the only class of phytohormones that has been implicated in affecting the floral transition. For example, there is evidence from studies on *S. alba* that long-distance signaling by cytokinins might play a role in the transition to flowering in response to inductive photoperiods (reviewed in Bernier et al., 1993). As discussed above, inductive photoperiods cause the rapid and transient export of sucrose from the leaves to both the shoot and root meristems. In the root, this sucrose leads to the export of cytokinin, primarily zeatin riboside, to the shoot and leaves, presumably via the xylem. Subsequently, another cytokinin, isopentenyladenine riboside, moves out of the leaves, and some makes its way to the shoot apex, where its levels increase within 16 hr of induction (Bernier et al., 1993).

The relative importance of the cytokinin and sucrose fluxes to the floral transition in Arabidopsis remains to be established. Application of cytokinins provokes a phenotype similar to that of *deetiolated 1* mutants—early flowering and severe pleiotropic effects on growth (Chory et al., 1994). *emf2* has been shown to be allelic (Z.R. Sung, personal communication) to the cytokinin resistance mutant *cyr1* (Deikman and Ulrich, 1995), but the apparent lack of mutations that implicate cytokinins in flowering may be due to a high degree of redundancy in the genes involved. Alternatively, the mutant phenotypes may be so pleiotropic that such mutants have not been classified as cytokinin mutants.

In addition to GAs and cytokinins, other phytohormones, such as abscisic acid (ABA), ethylene, and polyamines, may be involved in flowering under certain circumstances and in some species (Martínez-Zapater et al., 1994). The ethylene-insensitive mutant *ein2* is slightly delayed in flowering, and ABA-deficient mutants flower somewhat early under noninductive photoperiods (Martínez-Zapater et al., 1994), suggesting a role for ethylene and ABA in floral promotion and repression, respectively.

GENETIC INTERACTIONS THAT CONTROL THE FLORAL TRANSITION

The genetic interactions that control the floral transition in Arabidopsis have been described in a model that is constantly updated and revised as new data become available (Figure 2; see, e.g., Schultz and Haughn, 1993; Martínez-Zapater et al., 1994; Coupland, 1995; Yang et al., 1995; Koornneef et al., 1998a). This model fits well with the multifactorial control

model, which was developed on the basis of physiological analyses of flowering time (Bernier, 1988). Its essential feature is that the time at which flowering occurs is determined by antagonism between the promotive action of parallel pathways that monitor developmental age and environment and the repressive action of floral inhibitors. The promotive pathways are functionally redundant, explaining why no single mutation that prevents flowering has yet been found.

How the long-day, autonomous promotion, and GA pathways integrate to activate the meristem identity genes is one of the most active areas of research in this field. Quantitative increases in *LFY* expression are clearly required, with flowering occurring only after a threshold concentration of LFY has been reached (Blázquez et al., 1998). Expression of *AP1* is more qualitatively linked to floral determination (Hempel et al., 1997). Unlike *LFY* and *AGL-8*, expression of *AP1* is upregulated after the point of floral determination. The connection between the flowering-time genes and *LFY* has been directly addressed (Blázquez et al., 1998; Nilsson et al., 1998). Indeed, *CO*, *GI*, *FCA*, *FVE*, *GA1*, and *GAI* all play a role in activation of *LFY* (Figure 2) and are required to some extent for full expression of *LFY* function. In contrast, *FWA*, *FE*, and *FT* appear to be necessary for plants to respond to *LFY* expression (Nilsson et al., 1998). *FT* has recently been cloned independently by T-DNA tagging (Araki et al., 1998) and activation tagging (D. Weigel, personal communication); it encodes a protein with pronounced similarity to another meristem identity gene, *TFL1* (Bradley et al., 1997). Despite their similarity, *TFL1* and *FT* have opposing functions, with one repressing and the other promoting flowering.

Genetic analyses by Ruiz-García et al. (1997) have distinguished *FWA* and *FT* from the other flowering-time genes, and it has been proposed that these two genes function to activate *AP1* in a pathway that runs parallel to the pathway leading to *LFY* activation (Figure 2). This separation of *FT* and *FWA* was also observed by Roldán et al. (1997) in their study of the sucrose-dependent acceleration of flowering in Arabidopsis flowering-time mutants (see The Role of Carbohydrates in Flowering, above). Thus, *FWA* and *FT* appear to act as intermediaries between some of the other floral promoters and floral meristem gene activation (Figure 2). How the many known floral meristem genes fit into this picture remains to be seen, but it is clear that different promotive pathways converge to redundantly activate a large set of floral meristem identity genes, which are themselves at least partially redundant in function. As stated previously, this area has been extensively reviewed recently and so is not covered in great detail here (see Figure 2; Koornneef et al., 1998b; Piñeiro and Coupland, 1998).

PERSPECTIVES

In summary, very rapid progress is being made in elucidating the molecular control of the floral transition. The next phase of the work will require the use of genetic screens designed, for example, to identify suppressors and enhancers of existing mutations. Creative genetic strategies that take advantage of the ability to constitutively express individual flowering-time genes or that use specific mutant backgrounds will help to identify both genes that operate downstream in the same pathway and genes with redundant functions. As more flowering-time genes are cloned, biochemical and cellular characterization of their products will become increasingly important. Several flowering-time genes that have already been cloned appear to encode regulators of gene expression (Table 2); identification of the upstream and downstream targets of these gene products will help to establish their regulatory role and, perhaps, to confirm genetically defined steps in the various signaling pathways.

As the genes controlling flowering time in Arabidopsis become better defined, an important question will be to address how they correspond to genes that regulate flowering time in other species. A focused effort on comparative mapping will be required to establish the potential correspondence of different genes in different species. With this goal in mind, we have assembled a list of possible orthologs from Arabidopsis, pea, sugar beet, barley, and wheat (all vernalization-responsive, quantitative, long-day plants), based on the physiological characteristics of the mutants or allelic variants and genetic dominance for late- or early-flowering phenotypes (Table 3).

Establishing correspondence among these different genes would clearly accelerate their cloning, and it would also provide useful information on gene function in Arabidopsis. The ability to combine grafting with genetic analysis in peas has provided important information on the role of the flowering-time genes. For example, the *Gigas* gene product is involved in the production of a graft-transmissible floral promoter, whereas the products of *Late flowering* and *Vegetative 2* are not graft transmissible and are thought instead to alter the threshold sensitivity of the meristem to the transmissible signals. Determining whether *Gigas*, *Late flowering*, and/or *Vegetative 2* correspond to *FCA* and/or *FRI* would significantly add to our understanding of the function of these Arabidopsis genes. Although gene function may have diverged during evolution, the identification of orthologs in different species would inform a working model, which could then be tested.

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Table 3. Possible Orthologs of Arabidopsis Flowering-Time Genes[a]

Class of Gene Product	Species: Arabidopsis	Brassica[b]	Pea	Sugar Beet	Barley	Wheat
Promotes flowering independent of photoperiod	*FCA* *FVE* *LD*	[c]	*Gigas*	*B*	*Spring habit 2*	*Vernalization 1*
Promotes flowering in response to inductive photoperiods	*CO* *GI*	Bn *LG2, 8*			*Photoperiod H1* (Igri)	*Photoperiod 1* and *2*[d]
Inhibits flowering in response to non-inductive photoperiods	*ELF3*	–	*Sterile node* *Day neutral* *Photoperiod response*	–		
Inhibits flowering and confers vernalization requirement	*FRI* *FLC*	Bn *VFN1* Br *VFR1* Bn *VFN2* Br *VFR2*	*Late flowering* *Vegetative 2*	–	*Spring habit 1*	*Group 6* gene(s)

[a] Restricted to vernalization-responsive, quantitative, long-day plant species. See Bezant et al. (1996); Snape et al. (1996); Laurie (1997); Law and Worland (1997); and Osborn et al. (1997).
[b] QTLs. Bn, *Brassica napus*. Br, *Brassica rapa*.
[c] None detected.
[d] In contrast to *CO*, *Photoperiod 1* and *Photoperiod 2* confer early flowering in both long days and short days. They may represent dominant gain-of-function alleles (Laurie, 1997).

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A Sequence-ready Contig Map of the Top Arm of *Arabidopsis thaliana* Chromosome 3

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Abstract

A fine physical map of the top arm of *Arabidopsis thaliana* chromosome 3 has been constructed by ordering P1, TAC and BAC clones using the sequences of a variety of DNA markers and end-sequences of clones. The marker sequences used in this study were derived from 58 DNA markers, 93 YAC end-sequences, and 807 end-sequences of P1, TAC and BAC clones. The entire top arm of chromosome 3, except for the centromeric and telomeric regions, was covered by a single contig 13.3 Mb long. This fine physical map will facilitate gene isolation by map-based cloning experiments as well as genome sequencing of the top arm of chromosome 3. The map and end-sequence information are available on the web site KAOS (Kazusa *Arabidopsis* data Opening Site) at [http://www.kazusa.or.jp/arabi/].

Key words: *Arabidopsis thaliana*; chromosome 3; physical map; sequence-ready contig map

1. Introduction

Arabidopsis thaliana is an excellent model organism for analysis of the complex processes in plants using classical and molecular genetic techniques,[1] and intensive efforts have been made for the isolation of *Arabidopsis* genes of biological importance using map-based cloning strategies. This plant has also been chosen as a target for sequencing of the entire genome,[2,3] because the estimated genome size is the smallest among known higher plants partly due to the lower content of repetitive sequences.[4] Under these circumstances, the construction of a complete physical map of the *Arabidopsis* genome should be greatly advantageous for cloning the genetic loci of interest as well as sequencing the entire genome. According to this view, yeast artificial chromosome (YAC)-based physical maps of chromosome 2,[5] 3,[6] 4[7] and 5[8] of *A. thaliana* have been reported. We also constructed a fine physical map of the entire chromosome 5 by ordering CIC YAC,[9] P1,[10] TAC[11] and BAC[12,13] clones to support the initial phase of our sequencing project,[14] and sequence analysis of this chromosome is in progress.[15] In the second phase of our project, we focused our target on the top arm of chromosome 3 in accordance with the international agreement of the Arabidopsis Genome Initiative.[2] To aid this project, we previously constructed a physical map of the entire chromosome 3 mostly covered by CIC YAC clones.[16] To advance the sequencing project, however, a fine physical map based on a single contig of clones with appropriate sizes for sequencing is essential. We present here a physical map of the top arm of chromosome 3 which was constructed by ordering the clones from P1, TAC and BAC libraries using the sequence information on the various DNA markers and end-sequences of clones.

2. Materials and Methods

2.1. Libraries

The following four kinds of libraries made from the genome of *A. thaliana* ecotype Columbia were used for the construction of the map: P1[10] and TAC[11] libraries from Mitsui Plant Biotechnology Research Institute, TAMU BAC[12] and IGF BAC[13] libraries.

2.2. DNA markers

The DNA markers used for the designation of PCR primers were: restriction fragment length polymorphism (RFLP) markers, cleaved amplified polymorphic sequence (CAPS) markers, single-stranded length polymorphism (SSLP) markers, expression sequence tags (EST)

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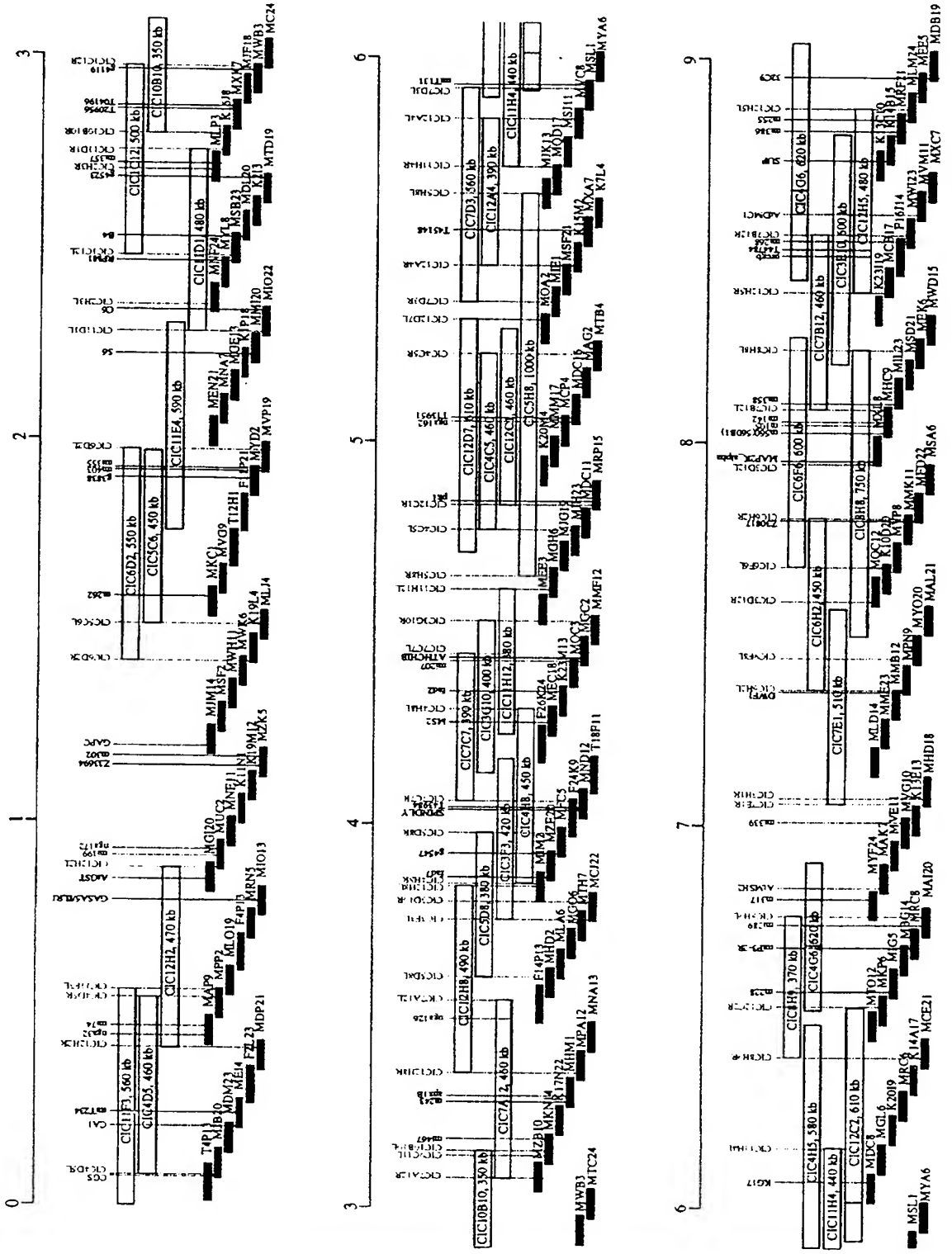

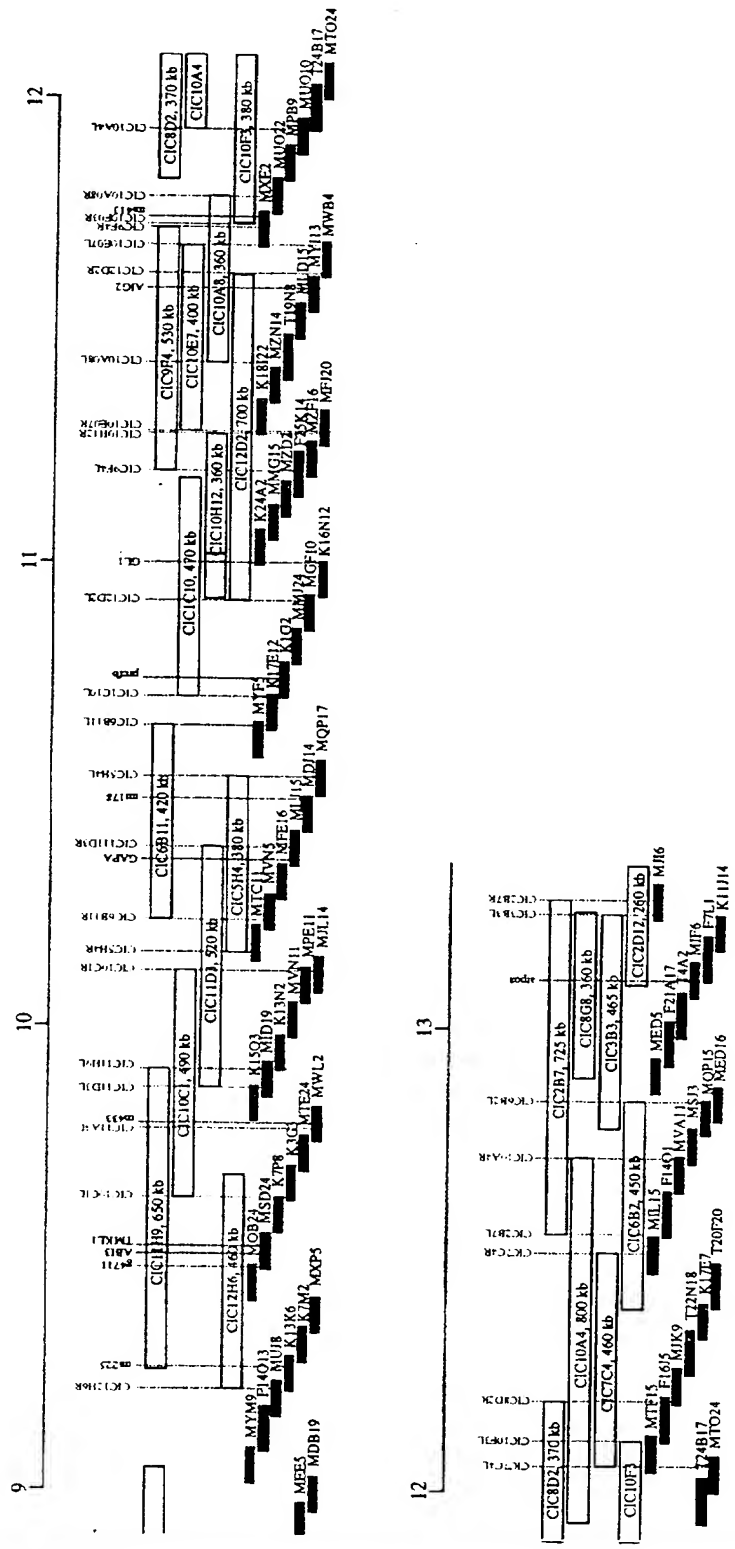

Figure 1. The physical map of the top arm of chromosome 3. The name and size of CIC YAC clones are indicated in wide open boxes with lengths reflecting the size of each clone. The putative chimeric parts of the YAC clones are indicated by shaded boxes. P1, TAC and BAC clones consisting the minimum tiling path are represented by thin open boxes of blue, green and red, respectively, below the wide boxes indicating the YAC clones. The names of bacterial clones are indicated at the right side of the bars as P1 (M####), TAC (K####), IGF BAC (F####), TAMU BAC (T####). RI markers (vertical red letters), other DNA markers (vertical black letters) and YAC end markers (vertically green letters) are shown on the top of each contig, and the approximate location of each marker on the clone is indicated by the vertical line crossing the clone(6).

markers and cloned genes. The species of respective markers and their sources are given in Table 1 of the previous paper.[16] The sequence information of YAC end markers used in this study are available through our web site at (http://www.kazusa.or.jp/arabi/endseq/).

2.3. Screening and clone analysis

To construct the physical map by ordering clones along the chromosome, PCR screening of DNA pools derived from each library was performed. The method of end sequence analysis of positive clones was described in the previous paper.[14] Some of the BAC end-sequence information used in this study could be retrieved from the BAC end-sequence database maintained at The Institute for Genomic Research (http://www.tigr.org/tdb/at/atgenome/bac_end_search/bac_end_search.html). The sizes of CIC YAC clones, the reported size of which was prural[9] or inconsistent with the allocated size on the physical map, were confirmed by PFG electrophoresis (CHEF Mapper system; Bio-Rad, Richmond, CA) followed by Southern hybridization using the probes amplified from the sequences of the corresponding DNA markers.

3. Results and Discussion

3.1. Construction of physical map

Among the four libraries, P1 and TAC libraries were mainly used for the construction of the contig map, because the clones in these libraries have an average insert size of 80 kb, which is suitable for large-scale sequencing and the construction of a high-resolution physical map. To increase the efficiency and specificity of screening, we adopted the PCR screening method using 3-dimensional DNA pools for the identification of neighboring overlapping clones within each library. As the first step of screening, P1 and TAC clones harboring a variety of DNA markers and YAC end markers used in the previous study[16] were isolated by PCR. Then, the end-sequences of these "seed clones" were determined to create new sequence-tagged sites (STSs), and the primers designed from such end-sequences were used for the following screening. These primers were also used to anchor the isolated clones onto the corresponding YAC tiling path, and the location and authenticity of the clones were confirmed. As the result of screening of P1 and TAC libraries, a physical map consisting of 11 contigs was obtained. All the remaining gaps were then closed by walking which uses IGF and TAMU BAC libraries.

Consequently, the entire top arm of chromosome 3 could be covered by a single contig of P1, TAC and BAC clones. This final contig was 13.3 Mb in length and was composed of 399 P1, 121 TAC and 76 BAC clones. The redundancy of coverage was 3.69. The size estimation was done by putting individual clones on the mid-point of its allocated position and using the average insert size, 80 kb for P1 and TAC clones and 100 kb for BAC clones. A minimum tiling path of the chromosome 3 top arm, composed of 211 P1, TAC and BAC clones, is shown in Fig. 1. Thirty-one RI markers whose genetic distances had been derived from up to 101 lines of a recombinant inbred mapping population[17] were precisely localized along the physical map at positions between 3.76 cM (CGS) and 52.32 cM (atpox). This indicates that most of the gene-rich regions of the chromosome 3 top arm were covered by the constructed contig. Through this study, a total of 807 end sequences were determined and anchored on the physical map, which produces a marker density of 1 STS per 16.5 kb.

3.2. Comparison of genetic and physical maps

A comparison of the genetic map and the fine physical map constructed in this study is shown in Fig. 2. The order of genetic markers in the contig well coincides with that of the physical map except for the following markers: 1) mi467 (15.58 cM on the RI map) and mi357 (16.15 cM) were mapped on the 3.2 Mb and 2.7 Mb positions respectively in the physical map, 2) mi358 (53.18 cM on the RI map) was mapped on the 8.1 Mb position between mi142 (29.23 cM) and mi268 (30.79 cM).

The ratio of the physical to genetic distance between markers varied significantly along the chromosome (Fig. 2). Compared to the previous map mostly composed of YAC clones,[16] the resolution of physical map was markedly increased. Nevertheless, the pattern of the ratio variation along the chromosome was almost consistent. Cold spots of recombination are observed near the middle of the contig (mi403 to mi289) in addition to the bottom region adjacent to the centromere. A similar pattern of recombination frequency along the chromosome arm has also been reported for chromosomes 4[18] and 5.[7]

3.3. Comparison of cytogenetic and physical maps

The centromeric region of chromosome 3 has been localized at the position of marker RCEN3 (53.74 cM) in the RI map. Although we did not map this marker on our fine physical map, it seems likely that the contig reached the boundary between the heterochromatin and euchromatin, because the YAC clone CIC2D12 allocated in the most distal position contained the 180-bp repeats[19–21] characteristic of centromeric regions, and the end-sequence of the P1 clone MJI6 allocated near the end of CIC2D12 contained other kinds of highly repetitive sequences (unpublished observation). The physical distance of the telomere to the top end of the contig has not been estimated.

The map and end-sequence information presented in this study are available on the web site KAOS (Kazusa *Arabidopsis* data Opening Site) at [http://www.kazusa.or.jp/arabi/].

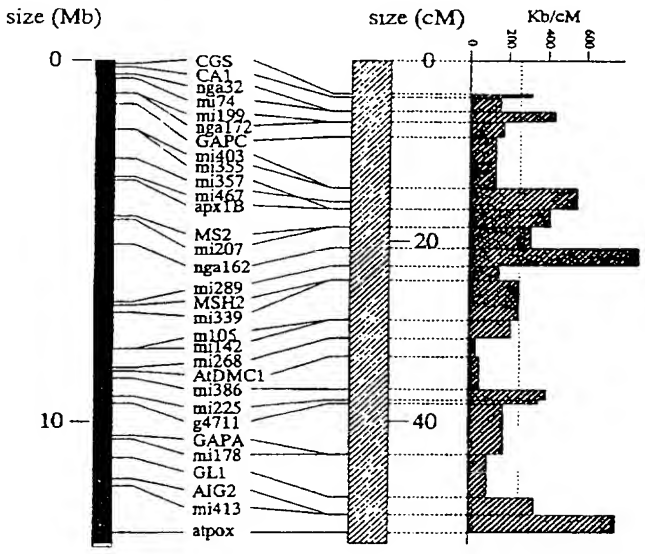

Figure 2. The alignment of the physical and RI maps of the chromosome 3-top arm and the ratio of the physical (Kb) and genetic (cM) scales along the RI map Thirty-one markers which have been mapped on the RI map were assigned on the physical map according to Fig. 1. The gray box at the left represents the physical map. The size of the physical map (Mb) is shown on the left side. The shaded box at the middle indicates the RI map with the size (cM) on the right. The ratio of the physical scale (Kb) to the genetic scale (cM) was calculated from the physical and genetic distances, and the average ratio is shown by vertical broken lines.

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Arabidopsis mutants showing an altered response to vernalization XP-002139145

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Summary

Flowering in many plant species is accelerated by a long period of cold temperature, known as a vernalization period. This research investigates how this cold temperature signal is perceived by plant cells and the mechanism by which it influences the transition to flowering. Mutagenesis of the late-flowering, vernalization-responsive, *Arabidopsis* mutant, *fca*, has yielded five independent mutations (termed *vrn* mutations) conferring an altered vernalization response. Allelism tests showed that these mutations fall into at least three complementation groups defining three loci named *VRN 1, 2* and *3*. The *vrn1* and *vrn2* mutations did not affect the acclimation response as judged by expression of cold-induced transcripts and freezing tolerance assays. *vrn1-1* affected the short-day vernalization response of Landsberg *erecta* and reduced the vernalization response of other late-flowering *Arabidopsis* mutants. The acceleration of flowering by GA_3 was not affected by *vrn1-1*. The *VRN 1* locus was mapped to chromosome 3.

Introduction

In many plant species the transition from vegetative to reproductive growth is strongly influenced by environmental conditions, such as cold temperature and day length. However, the molecular mechanisms that regulate this transition are still largely unknown (Bernier, 1988; Evans, 1960; Napp-Zinn, 1987). *Arabidopsis thaliana* provides an excellent system with which to carry out a molecular genetic analysis of the control of the floral transition as the flowering time is affected by environmental conditions and many loci and mutations that influence *Arabidopsis* flowering time have been identified (reviewed in Haughn *et al.*, 1995; Martinez-Zapater *et al.*, 1994). In addition, genes known only by their mutant phenotype can be cloned using a map-based cloning strategy. There are a number of mutations conferring a late-flowering phenotype, the majority of which have been isolated in the Landsberg *erecta* (L*er*) ecotype. The late-flowering mutations fall into 12 complementation groups and result in differential responses to cold temperature (vernalization) and day length (Martinez-Zapater and Somerville, 1990; Koornneef *et al.*, 1991). These mutations are being extensively characterized (Chandler and Dean, 1994; Martinez-Zapater *et al.*, 1995) as it is likely they identify genes involved in regulating the timing of the floral transition in response to developmental and environmental (internal and external) signals. The genes corresponding to the late-flowering loci have been or are being cloned (Lee *et al.*, 1994; Putterill *et al.*, 1995) and double mutants are being constructed with some of the many mutations previously isolated in the L*er* ecotype. This allows interactions between late-flowering mutations and other hormonal or meristem identity genes to be analysed, without complications of modifications to the flowering time phenotype from alleles of modifier loci present in other ecotypes.

A vernalization treatment (2–8 weeks at 4°C) results in accelerated flowering for the majority of the late-flowering mutants, with the *fca* mutant showing the greatest response (Koornneef *et al.*, 1991; Martinez-Zapater and Somerville, 1990). The vernalization response is thought to be perceived by dividing cells of the meristem (Metzger, 1988; Schwabe, 1954) and there is a quantitative relationship between the length of the vernalization period and the acceleration of flowering time (Napp-Zinn, 1987). In addition, the effect of vernalization treatment may be transmitted through mitosis but not through meiosis (Evans, 1960). Recently, Burn *et al.* (1993) have proposed that DNA methylation is involved in the vernalization mechanism.

In order to fully elucidate the mechanism of perception of the cold temperature signal and to understand how this accelerates the transition of the meristem to floral development, we have isolated and characterized *Arabidopsis* mutants having a reduced response to vernalization. We chose to mutagenize the late-flowering *fca* line as *fca* plants exhibit a consistently strong vernalization response and the *fca* mutation is in the well-characterized L*er* background. Five independent mutants, falling into at least three complementation groups were isolated from 36 000 M_2 seedlings. Here we present a characterization of two of the mutants in terms of their flowering time, vernalization response, and their effect on acclimation, another cold temperature response. The *vrn1* mutation was mapped to chromosome 3. *vrn1* was further characterized to

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determine the effect of GA_3 on its flowering time and to study its effect on the vernalization response of other late-flowering mutants.

Results

Isolation of mutations that reduce the vernalization response of fca-1

To identify mutations in genes needed for perception of or response to *vern*alization (*vrn* mutations), *fca-1* seed was mutagenized with EMS and 120 vernalized progeny from each of 300 bulked M_2 families (36 000 M_2 plants in total) were grown and individuals flowering later than the vernalized *fca-1* controls identified. Following progeny testing of M_3 seed from 65 such plants, 18 individuals were selected which showed a heritable and significant late-flowering phenotype following vernalization. A mutation that specifically disrupts the vernalization response of *fca* plants should cause vernalized *fca* plants to flower late, but should not cause late-flowering on its own in the L*er* background. Certain late-flowering mutants, such as *co*, *gi*, etc., flower later than *fca* or L*er* after vernalization (Koornneef *et al.*, 1991), and *fca* plants containing these mutations would also be isolated from a screen for mutant *fca* plants flowering late after vernalization. It was therefore necessary to determine whether the EMS mutations specifically affected the vernalization response or represented non-vernalization-responsive late-flowering mutations. This was performed by backcrossing the mutants to L*er* and scoring the F2 population for flowering time in the absence of a vernalization treatment (illustrated in Figure 1). Depending on the segregation ratios obtained, predictions can be made about.the type of mutation identified. A segregation ratio of 3:1 early- to late-flowering plants would indicate that only *fca* was segregating to cause late-flowering without vernalization and that the new mutation caused late-flowering after vernalization by reducing the vernalization response of *fca*. Such mutations were classified as *vrn* mutations. A 3:1 segregation ratio would also result from a second mutation conferring non-vernalization responsive late-flowering that was tightly linked to *fca-1*. However, no mutation causing late-flowering, closely linked to *fca*, has been identified by previous screens. A segregation ratio of 9:7 early to late-flowering would indicate that the new mutation was recessive, also conferred late-flowering, and was unlinked to *fca-1*. Intermediate ratios would indicate the new mutation conferred late-flowering but was linked to varying degrees to *fca-1*. Table 1 shows the segregation for flowering time in the non-vernalized F_2 progeny of the 18 putative *vrn* mutants crossed to L*er*. F_2 progeny from seven of the crosses segregated early- to late-flowering individuals with a ratio not significantly different from 3:1. Six gave progeny segregating with a ratio not significantly different from 9:7. Ratios for F_2 populations derived from 47A2,49 and 35D1,19, 30D2,52, 26A2,52 and 50A1,41 were significantly different from 3:1 and 9:7 early to late-flowering. Apart from 47A2,49, only mutants segregating 3:1 when crossed to L*er* were analysed further.

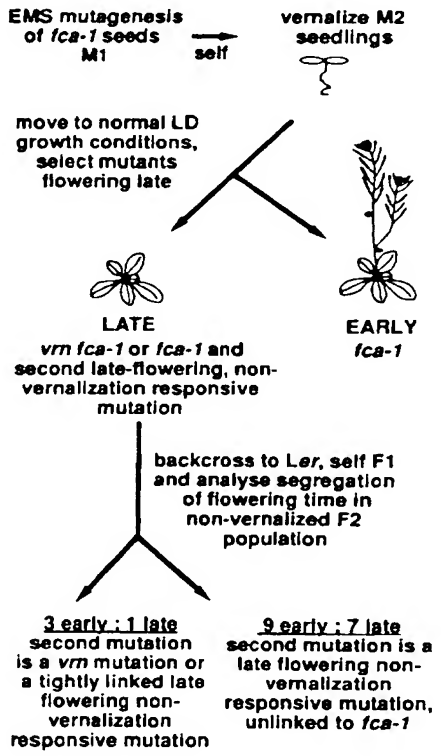

Figure 1. Scheme for isolation of mutations reduced in vernalization response.
Following a vernalization treatment late-flowering mutants were isolated from an *fca-1* M_2 population. The late-flowering individuals were crossed to L*er* plants and the flowering time of F_2 plants was scored. A ratio of 3:1 early to late-flowering, in the absence of vernalization, is indicative of a line carrying only one late-flowering mutation, namely, *fca-1* and a second mutation specifically affecting the vernalization response. It could also result from the presence of a second late-flowering mutation, tightly linked to *fca*, that does not respond to vernalization.

vrn *mutations are recessive and represent at least three distinct loci*

The *vrn* mutants were backcrossed to *fca-1* and the flowering time in vernalized F_2 progeny scored. The mutants all segregated with ratios not significantly different to 3:1 early to late-flowering (see Table 2) indicating that the mutations are completely recessive.

The putative *vrn*, *fca* mutants were crossed to each other and the flowering time of F_1 plants and F_2 progeny scored. The segregation of flowering time in the F_2 generation is shown in Table 2. All four mutations isolated from the same M_2 family (47A)–47A1,7,7, 47A2,19, 47A2,39 and

Table 1. Segregation for flowering time in F2 progeny from putative *vrn* mutants backcrossed to L*er*

Mutant	Segregation ratio (early:late)	$\chi^2$3:1	$\chi^2$9:7
47A1,7,7	123:35	$P > 0.05$	–
47A2,19	230:70	$P > 0.05$	–
47A2,39	275:72	$P > 0.05$	–
47A2,49	249:48	$P > 0.05$	–
8A1,20	180:59	$P > 0.05$	–
39A1,1	233:67	$P > 0.05$	–
57D1,33	123:53	$P > 0.05$	–
34C1,23	136:43	$P > 0.05$	–
37C1,9	172:128	–	$P > 0.05$
15B2,25	166:133	–	$P > 0.05$
38A2,45	146:143	–	$P > 0.05$
5D2,12	184:114	–	$P > 0.05$
5D2,17	171:128	–	$P > 0.05$
9A1,46	50:29	–	$P > 0.05$
35D1,19	168:35	$P < 0.02$	–
30D2,52	164:75	$P < 0.05$	–
26A2,52	207:91	$P < 0.05$	–
50A1,41	114:65	–	$P < 0.05$

Plants having leaf numbers greater than those of L*er* control plants were scored as late flowering. F_2 plants were not vernalized.

47A2,49 were found to be allelic and define the locus *VRN1*. It is likely that they all represent the same mutagenic event. The F_2 progeny of crosses between 8A1,20 and members of the 47A family segregated 9:7 early to late and 8A1,20 thus represents a second unlinked locus, *VRN2*. 8A1,20 and 34C1,23 were found to be allelic. Since 8A1,20 and 34C1,23 were isolated from different M_2 families they represent independent alleles of the *VRN2* locus and have been designated *vrn2-1* and *vrn2-2*, respectively. The mutations 39A1,1 and 57D1,33 were not alleles of either *VRN1* or *VRN2* and thus represent at least one other independent locus (*VRN3*). For the crosses 34C1,23 × 47A1,7,7 and 39A1,1 × 47A1,7,7, the F_2 had χ^2 (9 early:7 late) $P < 0.05$, however, the large number of early plants segregating in both crosses clearly indicates that the mutations are not allelic. At the time the complementation tests were performed, other mutations induced by the EMS mutagenesis were segregating in the background, and it is possible that these mutations resulted in a distortion of the 9:7 F_2 ratio. All *vrn* mutations are being further backcrossed to L*er* to eliminate such background mutations. The nomenclature of the mutant alleles is: 47A1,7,7 = *vrn1-1*; 8A1,20 = *vrn2-1*; 34C1,23 = *vrn2-2*; 57D1,33 = *vrn3-1*; 39A1,1-allelism not yet determined.

vrn1-1 *and* vrn2-1 *show different responses to vernalization*

The initial screens for *vrn* mutations were conducted under greenhouse conditions. In order to accurately assess the flowering phenotype of *vrn1-1 fca-1* and *vrn2-1 fca-1*, the plants were grown under controlled conditions and the total leaf number (LN) at flowering was measured for vernalized or non-vernalized plants. The results are summarized in Table 3. In the absence of vernalization *vrn1-1 fca-1* plants flowered at the same time as *fca-1* plants, however, after a vernalization treatment they flowered considerably later. The *vrn1-1* mutation reduced the vernalization response of *fca-1* by 42%. *vrn2-1 fca-1* plants flowered later than *fca-1* plants in the absence of vernalization. After vernalization they flowered with 31 leaves, as late as *fca-1* without vernalization, suggesting that *vrn2-1* confers a complete loss of vernalization response. However, the later flowering of non-vernalized *vrn2-1 fca-1* plants implies that *vrn2-1* does not simply confer a complete loss of response to vernalization.

Table 2. Dominance tests and complementation analyses—ratio of early to late flowering in F_2 progeny

Cross (early:late)	Segregation ratio	χ^2(3:1)	χ^2(9:7)
47A1,7,7 × *fca-1*	144:36	$P > 0.05$	–
8A1,20 × *fca-1*	143:37	$P > 0.05$	–
57D1,33 × *fca-1*	129:41	$P > 0.05$	–
39A1,1 × *fca-1*	126:34	$P > 0.05$	–
47A1,7,7 × 47A2,19	0:106	–	–
47A1,7,7 × 47A2,39	0:111	–	–
47A2,19 × 47A2,49	0:116	–	–
47A1,7,7 × 8A1,20	131:108	–	$P > 0.05$
47A2,19 × 8A1,20	70:50	–	$P > 0.05$
34C1,23 × 8A1,20	0:356	–	–
34C1,23 × 47A1,7,7	154:71	–	$P < 0.01$
57D1,33 × 8A1,20	141:96	–	$P > 0.05$
57D1,33 × 47A1,7,7	59:61	–	$P > 0.05$
39A1,1 × 8A1,20	164:124	–	$P > 0.05$
39A1,1 × 47A1,7,7	115:64	–	$P < 0.05$
57D1,33 × 39A1,1	n.d.	–	–

F_2 plants were vernalized for 8 weeks at 4°C and scored for segregation of early- to late-flowering plants. All *vrn* mutants have *fca-1* in their background. Plants having a leaf number greater than vernalized *fca-1* control plants were scored as late flowering. F_2 ratios with $P > 0.05$ indicate segregation ratios consistent with either 3:1 or 9:7 early to late flowering.
n.d., not determined.

Table 3. Total leaf number for vernalized and non-vernalized plants

Genotype	–Vernalization	+Vernalization
L*er*	6.1±0.1	5.9±0.1
L*er fca-1*	27.2±1.0	8.1±0.3
vrn1-1 fca-1	29.3±1.6	17.0±0.9
vrn2-1 fca-1	46.1±2.6	31.8±1.8

Numbers are means of total leaf numbers ± SE for eight to 20 plants.

The vrn *mutations do not affect the acclimation response*

Cold temperature has a number of effects on the physiology of plants: in addition to causing earlier flowering, a cold treatment at 4°C for shorter periods of time than vernalization (up to 1 week) causes *Arabidopsis* plants to acclimate, and thus survive subsequent freezing temperatures (reviewed in Thomashow, 1994). Given the similarity of the initial environmental conditions necessary for both vernalization and acclimation we wanted to test whether the *vrn* mutants were impaired in their ability to acclimate. This was assayed in two ways, first, the induction of a number of transcripts previously shown to be strongly upregulated during an acclimation treatment was analysed. The expression of four cold-induced transcripts *COR15* (now designated *COR15a* (Thomashow, 1994)) *COR78, PHH7.2* and *PHH29* (Hajela *et al.*, 1990) was analysed following 4 days at 4°C. The results were similar for all four transcripts. Following acclimation, a very large increase in steady-state mRNA levels was seen in both L*er* and *fca-1* plants following acclimation, which was indistinguishable from the increase seen in *vrn1-1 fca-1* and *vrn2-1 fca-1*.

The second assay was a freezing tolerance assay: *fca-1*, *vrn1-1 fca-1* and *vrn2-1 fca-1* plants non-acclimated or acclimated at 4°C for 1 week were subjected to progressively lower freezing temperatures. The temperature was reduced at 2°C h^{-1}, the plants held at the final temperature for 6 h and then returned to 4°C with the same regime of increasing temperature. For all the genotypes, non-acclimated plants showed between 83–100% survival at –5°C but 0% survival at –6°C. For acclimated plants 85–100% of plants of all three genotypes survived temperatures down to –9°C (Jose Martinez-Zapater, personal communication).

Both assays thus demonstrate that the *vrn1-1* and *vrn2-1* mutations do not significantly impair the plant's acclimation response.

The VRN1 *locus maps to chromosome 3*

The chromosomal location of the *vrn1-1* mutation was determined by establishing linkage between the mutation and RFLP markers, either lambda clones (Chang *et al.*, 1988), or plasmid clones (provided by Robert Whittier, Mitsui Plant Biotechnology Research Institute, Japan). A homozygous *vrn1-1* plant was crossed to a line from the ecotype Wassilewskija (WS) carrying an *fca* mutation (a kind gift from R. Amasino, University of Wisconsin). F_1 plants were selfed to generate F_2 plants segregating for *vrn1-1* and RFLPs between L*er* and WS ecotypes. Seed was collected from 77 F_2 plants. This seed was used for progeny testing for the *vrn* genotype, and to grow plants used to obtain DNA for RFLP analysis. Analysis of the segregation pattern of 22 markers polymorphic between Landsberg *erecta* and WS indicated that *VRN1* mapped to the upper arm of chromosome 3. Finer analysis with markers mapping on chromosome 3 defined the interval in which *VRN1* mapped as being between *mi207* and *mi399*.

vrn1-1 *reduces the vernalization response of all the vernalization-responsive late-flowering mutants*

The *vrn1-1* mutation had no effect on the flowering time of non-vernalized *fca-1* and so appears to specifically disrupt the perception of, or response to, vernalization. It is also unlinked to the *FCA* locus and so could definitively be classified as a *vrn* mutation. It was of interest to see whether the *vrn-1* mutation could disrupt the vernalization response of other vernalization-responsive, late-flowering mutants of *Arabidopsis*. In order to do this, it was necessary to segregate the *vrn1* mutation away from *fca-1*. A *vrn1-1 fca-1* homozygous plant was crossed to wild-type L*er*, and individual early-flowering F_2 plants were selected. F_3 seed from selfed F_2 plants was collected and sown and the flowering time monitored in F_3 populations. Those F_3 populations not segregating any late-flowering plants represented progeny of F_2 plants which were homozygous for the wild-type *FCA* allele. Wild-type L*er* plants only show a minimal vernalization response in long-day photoperiods, however, that response is much greater in short-days (mean LN non-vernalized approximately 28, mean leaf number vernalized approximately 22, Chandler, unpublished data). In order to select a line carrying the *vrn1* mutation, 20 F_4 seed from each F_3 plant homozygous for the wild-type *FCA* allele were vernalized, and grown under a short-day photoperiod. One F_4 family segregated later flowering individuals with a ratio of 3 early: 1 late. Late-flowering individuals were selected and selfed to confirm that they were indeed homozygous for the *vrn1-1* mutation.

This line was then backcrossed to *fca-1* and the flowering time after vernalization analysed in F_2 seedlings. Approximately 1/16 of the seedlings flowered late (Table 4). These experiments confirmed that we indeed had a line homozygous for the *vrn1-1* mutation that no longer carried the *fca-1* mutation.

This line was then crossed to the vernalization-responsive *Arabidopsis* late-flowering mutants *fve, ld, fwa, fe, fpa,* and *ft*. Segregation of flowering time, as assayed by LN, was analysed in vernalized F_2 progeny. The segregation ratio of early to late-flowering for each F_2 population is shown in Table 4. In all cases the ratio was not significantly different from 15:1, a ratio consistent with plants homozygous for both the late-flowering mutation and *vrn1-1* being late-flowering after vernalization. The whole experiment from the stage of crossing *vrn1-1* to the different late-flowering mutants was repeated and a similar set of ratios were found (data not shown).

Table 4. Segregation for flowering time in the F_2 from crosses of *vrn1-1* to late-flowering time in the F_2 from crosses of *vrn1-1* to late-flowering mutants

Cross	Segregation ratio (early:late)
fca-1 × *vrn1-1*	164:14
fve-1 × *vrn1-1*	172:7
ld-3 × *vrn1-1*	156:12
fwa-1 × *vrn1-1*	165:15
fe-1 × *vrn1-1*	170:9
fpa-2 × *vrn1-1*	170:10
ft-1 × *vrn1-1*	168:12

Plants were scored as late if they flowered with more or equal to the rosette leaf number (LN) of the non-vernalized late-flowering mutant control plants. Non-vernalized controls flowered with mean LN; *fca-1* = 12.5, *fve-1* = 11, *fwa-1* = 11, *fe-1* = 12.5, *fpa-2* = 11, *ft-1* = 10.5, L*er* = 5, *vrn1-1* = 8 and *vrn1-1 fca-1* = 12. Vernalized plants flowered with mean LN; *fca-1* = 6, *fve-1* = 5.5, *fwa-1* = 7.5, *fe1* = 8, *fpa-2* = 6, *ft-1* = 7. In all cases the χ^2 value gave a probability of more than 0.1, indicating that the results are in agreement with those expected for a 15:1 ratio of early:late plants.

Gibberellic acid still accelerates flowering time in vrn1 *mutants*

We have previously reported that GA_3 application to plants grown in agar medium significantly accelerated the flowering time of all the late-flowering mutants (Chandler and Dean, 1994). Gibberellins have been implicated in the vernalization response of a number of plants (Bernier, 1988; Hazebroek and Metzger, 1990; Hazebroek *et al.*, 1993). Thus, we were interested to see whether the flowering time of the *vrn* mutants could also be accelerated by GA_3. L*er*, *fca-1* and *vrn1-1 fca-1* plants were grown in sterile conditions with or without vernalization and in addition with or without GA_3 application. Vernalization of *fca-1* caused a greater acceleration of flowering time (LN 27–12) than GA_3 treatment (LN 27–17). *vrn1-1* plants showed a much reduced response to vernalization but an almost wild-type response to GA_3. These results indicate that the acceleration of flowering time by GA_3 is not impaired in *vrn1-1* mutants. When *fca-1* plants were given GA_3 and vernalized their flowering time was accelerated slightly compared with a vernalization treatment alone. This result suggests that GA_3 and vernalization may affect flowering time through different pathways. The flowering time of the *vrn1-1 fca-1* mutant in the double treatment was indistinguishable from that after a GA_3 treatment alone (Figure 2).

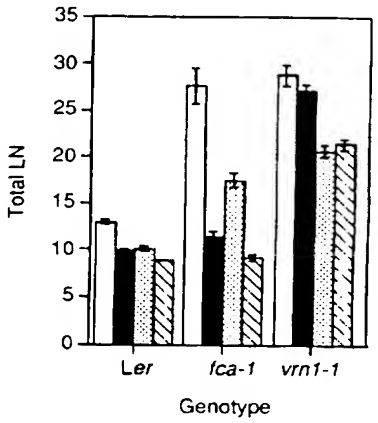

Figure 2. The effect of gibberellic acid on vernalization of L*er*, *fca* and *vrn1-1*. The four treatments shown for each genotype from left to right are: white, no vernalization no GA_3; black, vernalization; stippled, GA_3; hatched, vernalization plus GA_3. Values are means ± SE for 12–18 plants.

Discussion

Identification of mutations affecting the vernalization response of Arabidopsis

Through mutagenesis of the late-flowering *Arabidopsis* mutant *fca-1* we have identified three loci whose gene products play a role in the vernalization response. The *vrn1* mutation reduces the effect of vernalization on flowering time without altering flowering time of non-vernalized *fca-1* plants. The *vrn2* mutation reduces the vernalization response but also causes a delay in flowering of non-vernalized *fca-1* plants. The other mutations have yet to be characterized in detail.

The vernalization response exhibited by fca *in LD and* Ler *in SD are both disrupted by* vrn1

The *vrn1-1* mutation reduces the vernalization response such that the plants flower with approximately half the number of leaves of non-vernalized *fca-1*. This still allows *vrn1-1* homozygotes to be easily scored in a segregating population. The fact that some response to vernalization is still seen may be explained by *vrn1-1* not being a null allele or by redundancy, either in *VRN1* function or in the whole vernalization pathway. What is clear, however, is that the *vrn1-1* mutation affects the vernalization response in wild-type Landsberg *erecta* and all the vernalization-responsive late-flowering mutants. This therefore implies that the vernalization pathway present in the late-flowering mutants and in short-day photoperiods is likely to be the same—mutations in the late-flowering loci probably just uncover a need for the vernalization-responsive pathway. Landsberg *erecta* plants grown in long-day photoperiods probably undergo a vernalization response but the presence of saturating floral promotive gene products means no effect of vernalization on flowering time is visible. Several models (Dennis *et al.*, 1996; Martinez-Zapater *et al.*, 1994; Weigel, 1995) have postulated that the vernalization pathway is normally only significant in long-day-grown plants when a pathway known as the 'constitutive pathway' which involves loci such as *FCA*, *FVE* and *LD*, is knocked out.

vrn1 *and* vrn2 *do not affect the acclimation response*

Neither *vrn1-1* or *vrn2-1* altered the acclimation response of *Arabidopsis*, monitored either through freezing tolerance or induction of cold-induced transcripts. We have not so far analysed whether the *vrn* mutations alter seed dormancy. If there is a single mechanism for sensing low temperature then the *vrn* mutations identify loci downstream in the pathway, specific to the vernalization response. Alternatively, acclimation and vernalization are two completely separate pathways, in which the perception of the cold temperature is possibly perceived in very different parts of the plant. In support of this, there is evidence that the vernalization signal is perceived at the apex and cannot be translocated from elsewhere in the plant (Metzger, 1988; Schwabe, 1954). It is likely that the acclimation signal is perceived throughout the plant or rapidly translocated.

GA_3 *still accelerates flowering in* vrn1

The acceleration of flowering by GA_3 was as great in *vrn1-1 fca-1* as in *fca-1*. Thus, either the acceleration of flowering time by vernalization and GA_3 act through different pathways or the influence of GA_3 is downstream of the point of action of the *VRN1* gene product. This result does not resolve the question of whether gibberellins are involved in the vernalization response. The *ga1-3* mutant, which carries a deletion in the *GA1* locus encoding a product necessary for the first committed step in GA biosynthesis (Sun and Kamiya, 1994), was found not to respond to vernalization in short-day photoperiods (Wilson *et al.*, 1992). Also, in *Thlaspi arvense*, a crucifer related to *Arabidopsis*, vernalization dramatically increased the hydroxylation of the GA precursor kaurenoic acid to 7-OH kaurenoic acid at the shoot tip through a direct effect on the KA hydroxylase enzyme (Hazebroek and Metzger, 1990; Hazebroek *et al.*, 1993). However, arguing against the fact that gibberellins are involved in vernalization in *Arabidopsis*, we have found that an *fca-1 ga1-3* double mutant grown in continuous days responds well to vernalization (Chandler and Dean, unpublished data).

The availability of *vrn* mutations opens up the possibility of looking at the interaction of vernalization with many other factors that affect flowering time, for example, far-red irradiation (Bagnall, 1993; Martinez-Zapater and Somerville, 1990). It also opens up the possibility of map-based cloning. An understanding of the mechanism of vernalization and how the vernalization pathway interacts with all the other pathways influencing flowering awaits the identification of all the genes involved in the perception of the vernalization response and analysis of the biochemical function of their gene products.

Experimental procedures

Plant material and growth conditions

Plants grown in soil were sown directly on to a mixture of soil:grit:vermiculite (3:2:2), in plastipak pots, and grown either in the greenhouse (temperature controlled at 20°C for 16 h during the day; 15°C at night; daylight extended with a light supplement of about 70 Wm^{-2} from October until March), or in a Sanyo Gallenkamp controlled environment room under short-day or extended short-day growth conditions. Light conditions for the short-day room were 10 h illumination by 400 W Wotan metal halide power star lamps, PAR 113.7 μmol m^{-2} sec^{-1} and a R/FR ratio of 2.41. Light for the extended short-days was as for short-days (10 h) followed by 8 h illumination with Tungsten Halide lamps only, PAR 14.27 μmol m^{-2} sec^{-1}, and a R/FR ratio of 0.66. For plants grown on soil, individual plants were transferred to partitioned trays at about the four-leaf stage.

Plants grown in tissue culture were surface-sterilized by wetting with 70% ethanol, and soaking for 15 min in 5% (v/v) sodium hypochlorite with 0.2% Tween 20, followed by five rinses with sterile distilled water. Seeds were sown in petri dishes on AM media (1/2 MS salts (Flow labs); 0.5 mg l^{-1} nicotinic acid; 0.5 mg l^{-1} thiamine; 0.5 mg l^{-1} pyrridoxine; 100 mg l^{-1} inositol; 0.8% agar; 1% sucrose), and grown at 20°C in white fluorescent light (PAR 57.0 μmol m^{-2} sec^{-1}; R/FR ratio 7.5; photoperiod 16 h). When first true leaves began to expand, plants were transferred individually to plastic capped boiling tubes (one plant per tube), in 9 × 4 racks (Sigma Ltd). Racks were placed in trays made of black card, to a depth of 2 cm, to shade the roots.

For gibberellic acid treatments, a stock solution of GA_3 was filter-sterilized and added to media in petri dishes and boiling tubes at a final concentration of 10^{-4} M.

Plants were vernalized or acclimated at the seed stage, immediately after sowing on soil, or on agar. Vernalization was carried out for 8 weeks, and acclimation for 4 days, in an 8 h photoperiod (fluorescent light, PAR 9.5 μmol m^{-2} sec^{-1}, R/FR ratio 3.9) at a temperature of 5°C ± 1°C. Flowering time was assayed by total leaf number (LN), rosette plus cauline, counted once the bolt was more than 5 cm tall.

Mutagenesis experiment

A 0.5 g sample of *fca-1* seeds were soaked for 12 h in 0.3% ethyl methane sulphonate (EMS). Three thousand M_1 plants were grown in the greenhouse, and seed from 10 M_1 plants were bulk harvested to form M_2 families. One hundred and twenty plants from each of the 300 M_2 pools were vernalized on soil and grown in the greenhouse. Plants flowering with a greater LN than vernalized *fca-1* controls were selected as putative mutants having a decreased sensitivity to vernalization relative to *fca-1*.

RFLP mapping

For each F_3 family, DNA was isolated from 15–20 plants grown for 5 weeks in sterile liquid culture using a CTAB miniprep method (Dean *et al.*, 1992). Approximately 2 μg of genomic DNA were digested overnight with a fivefold excess of a restriction enzyme and fractionated by electrophoresis in 0.8% agarose gels at 0.5–2.0 Vcm^{-1}. Gels were blotted and cross-linked to Hybond-N filters (Amersham) according to the manufacturer's instructions. Filters were prehybridized for 3–5 h and hybridized (10^6–10^7 c.p.m. cm^{-3}) for 16–18 h at 65°C in a solution containing 5× SSC (1×

SSC is 150 mM NaCl, 15 mM sodium citrate), 0.5% SDS, 5× Denhardt's solution (0.1% Ficoll (400), 0.1% PVP (360) and 0.1% BSA (fraction 5)), and denatured salmon sperm DNA (0.0025% w/v). Filters were washed at 65°C in 2× SSC for 5 min, and twice in 2× SSC containing 0.1% SDS for 30 min. Filters were exposed to Kodak X-Omat XAR X-ray film for 1–5 days at −70°C with an intensifying screen. Filters were re-used several times, after removing the previous probe by washing at 45°C in 0.4 M NaOH for 30 min followed by 15 min at 45°C in 0.1× SSC, 0.1% SDS and 0.2 M Tris–HCl pH 7.5.

Linkage analysis was performed on a Macintosh computer, using the MapMaker programme (Lander *et al.*, 1987), a gift from S.Tingey (DuPont Co.).

RNA analysis

Total RNA was extracted using a method based on that of Logemann *et al.* (1987). Samples of between 0.5 and 3 g tissue were ground in liquid nitrogen using a mortar and pestle. Tissue was homogenized further by adding two volumes of guanidine buffer (8 M guanidine hydrochloride, 20 mM Mes, 20 mM EDTA and 50 mM mercaptoethanol at pH 7.0), and leaving for 1 h at room temperature. One volume of phenol/chloroform/isoamyl alcohol was added to the homogenate, which was then centrifuged at 1500 ***g*** for 10 min. The aqueous phase was collected and mixed with precooled 0.7 vol ethanol and 0.2 vol 1 M acetic acid and left overnight at −20° C. The precipitated RNA was recovered by pelleting at 1500 ***g*** for 10 min, and washed twice with sterile 3 M sodium acetate, pH 5.2 at room temperature, before centrifuging again at 1500 ***g*** for 5 min. The pellet was washed with 70% ethanol, and dissolved in 50–300 µl sterile water. RNA was denatured and separated by electrophoresis in formaldehyde-agarose gels and blotted according to Sambrook *et al.* (1989).

Acknowledgements

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Date of mailing (day/month/year) 22 May 2001 (22.05.01)	
International application No. PCT/GB00/03525	**Applicant's or agent's file reference** SMK/LP5872353
International filing date (day month year) 13 September 2000 (13.09.00)	**Priority date** (day month year) 17 September 1999 (17.09.99)
Applicant DEAN, Caroline et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

07 March 2001 (07.03.01)

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	LEVY YARON Y ET AL: "The transition of flowering" PLANT CELL,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 10, no. 12, December 1998 (1998-12), pages 1973-1989, XP002132682 ISSN: 1040-4651 the whole document	1-34
A	LIU YAO-GUANG ET AL: "Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 11, 25 May 1999 (1999-05-25), pages 6535-6540, XP002158766 May 25, 1999 ISSN: 0027-8424 the whole document	1-34
A	WILSON A ET AL: "ANALYSIS OF THE MOLECULAR BASIS OF VERNALIZATION IN ARABIDOPSIS THALIANA" SEMINARS IN CELL AND DEVELOPMENTAL BIOLOGY,GB,ACADEMIC PRESS, vol. 7, no. 3, 1996, pages 435-440, XP000609514 ISSN: 1084-9521 the whole document	1-34

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/GB 00/03525

A. CLASSIFICATION OF SUBJECT MAT[redacted]
IPC 7 C12N15/82 C12Q1/68 C07K14/415 C07K16/16 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, MEDLINE, BIOSIS, STRAND, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHANDLER ET AL: "arabidopsis mutants showing an altered response to vernalisation" PLANT JOURNAL,GB,BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 10, no. 4, 1996, pages 637-644, XP002139145 ISSN: 0960-7412 cited in the application the whole document ---	1-34
Y	SATO S ET AL: "A sequence-ready contig map of the top arm of Arabidopsis thaliana chromosome 3." DNA RESEARCH, (1999 APR 30) 6 (2) 117-21. ISSN: 1340-2838., XP000973646 figure 1 ---	1-34

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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3

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INTERNATIONAL SEARCH REPORT

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Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
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International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/ 03525	13/09/2000	17/09/1999
Applicant		
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[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

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   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

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2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   METHODS AND MEANS FOR MODIFICATION OF PLANT FLOWERING CHARACTERISTICS

5. With regard to the **abstract,**

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6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 6535–6540, May 1999
Plant Biology

Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning

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ABSTRACT **To accelerate gene isolation from plants by positional cloning, vector systems suitable for both chromosome walking and genetic complementation are highly desirable. Therefore, we developed a transformation-competent artificial chromosome (TAC) vector, pYLTAC7, that can accept and maintain large genomic DNA fragments stably in both *Escherichia coli* and *Agrobacterium tumefaciens*. Furthermore, it has the cis sequences required for *Agrobacterium*-mediated gene transfer into plants. We cloned large genomic DNA fragments of *Arabidopsis thaliana* into the vector and showed that most of the DNA fragments were maintained stably. Several TAC clones carrying 40- to 80-kb genomic DNA fragments were transferred back into *Arabidopsis* with high efficiency and shown to be inherited faithfully among the progeny. Furthermore, we demonstrated the practical utility of this vector system for positional cloning in *Arabidopsis*. A TAC contig was constructed in the region of the *SGR1* locus, and individual clones with *ca.* 80-kb inserts were tested for their ability to complement the gravitropic defects of a homozygous mutant line. Successful complementation enabled the physical location of *SGR1* to be delimited with high precision and confidence.**

Molecular genetic approaches have been applied to analysis and cloning of plant genes, particularly those involved in complex biological processes such as developmental regulation and gene expression cascades (1, 2). Genes defined by mutations are isolated by positional cloning as well as by DNA tagging. For positional cloning, efforts have been devoted to producing numerous sets of DNA markers and genomic DNA libraries from various plant species by using artificial chromosomes propagated in either yeast artificial chromosome (YAC) or bacteria artificial chromosome (BAC and P1) (3–5). Therefore, an initial mapping of target gene loci using DNA markers and subsequent isolation of large, overlapping genomic DNA fragments in the target region by chromosome walking or landing have become easier in several plant species, including *Arabidopsis thaliana* (1) and rice (6).

Proof of successful gene identification and cloning usually requires complementation of the mutant phenotype by transformation with a wild-type allele. The major drawback of positional cloning, however, is the difficulty of narrowing down the field of candidate clones to a manageable number for complementation testing. For fine-scale mapping of a mutation locus, it is usually necessary to analyze nearly a thousand progeny (usually F_2 plants) or even more if the locus falls in a "recombination cold spot," a chromosomal region of low recombination frequency (7). Furthermore, even low levels of misscoring during mapping (because of subtlety or incomplete penetrance of the mutant phenotype) will reduce mapping precision to the point that cloning becomes impractical. In addition, even after accurate mapping, present positional cloning procedures that use YAC or BAC clones require subcloning of many small fragments into a transformation-competent vector for complementation testing. In many cases, these steps are rate-limiting hurdles to positional cloning. Therefore, to accelerate positional cloning, it is highly desirable to exploit a strategy that streamlines complementation testing.

Plant transformation-competent vectors, such as the cosmid vector pOCA18 (8) and the λ-phage vector λTI2 (9), have been developed for construction of genomic libraries with inserts of 5–25 kb that are used for genetic complementation of mutants. The low cloning capacity of these vectors, however, limits their usefulness for efficient gene isolation by positional cloning. In a previous report on an *Arabidopsis* genomic DNA library prepared by using a P1 phage vector (10), we suggested that if large DNA fragments could be transferred directly from P1-based clones into plants, it would greatly accelerate positional cloning of plant genes. Recently, a 150-kb human DNA fragment was transferred into the tobacco genome by using a binary-BAC vector by *Agrobacterium*-mediated transformation (11, 12). Here we report a vector system for constructing transformation-competent artificial chromosome (TAC) libraries. Our results show that large genomic DNA fragments of *A. thaliana* cloned in a TAC vector can be maintained stably in both *Escherichia coli* and *Agrobacterium tumefaciens*, transferred with a high efficiency into the *Arabidopsis* genome, and faithfully inherited in the transgenic progeny. We also show that TAC clones carrying *ca.* 80-kb genomic DNA fragments of *A. thaliana* complement a gravitropic mutation at the *sgr1* locus.

MATERIALS AND METHODS

Construction of a TAC Vector. A TAC vector was constructed by using standard cloning procedures. Components of

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Abbreviations: TAC, transformation-competent artificial chromosome; BAC, bacteria artificial chromosome.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB020028).

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the vector were obtained from various plasmids in the minimum sizes possible. For blunt-end ligation, DNA fragments were treated with Klenow or T4 DNA polymerase. *E. coli* strain DH10B was used as the cloning host, and transformation of the bacterium was done by electroporation using Gene Pulser (Bio-Rad) according to the supplier's protocol. To create an initial backbone plasmid (pYL1), a 6.3-kb *Kpn*I-*Dra*III fragment carrying the P1 plasmid replicon of the P1 vector pAd10sacBII (4) was ligated to a 1.8-kb *Dra*III-*Ssp*I fragment from pACYC177 (New England Biolabs) containing the kanamycin-resistant marker gene (*NPT1*), in which the *Hin*dIII site had been destroyed (Y.-G.L., unpublished results). The P1 lytic replicon obtained from the P1 vector as a 1.7-kb *Ase*I fragment then was inserted into the *Aat*II site of pYL1 to yield pYL2. A T-DNA cassette was constructed in pBluescript II (Stratagene), which consists of components from pGA carrying the octopine-type left and right borders and the "overdrive" enhancer sequence (13) and pGDW32 carrying the hygromycin phosphotransferase gene (*HPT*) (14). This cassette was cloned into the *Bam*HI site of pYL2 to produce pYL3. The cloning-selection marker gene *sacB* isolated from pAd10sacBII was inserted between the nopaline synthase gene (*nos*) 3′ region and the left border to produce pYL4. An 8.1-kb *Bam*HI fragment containing the pRiA4 replicon (15, 16) was inserted into the *Bst*EII site of pYL4. Finally, a synthetic, double-stranded oligonucleotide containing rare-cutter sites and cloning sites (Fig. 1) was introduced between the *E. coli* promoter and the *sacB* gene. To create a unique *Hin*dIII cloning site in the vector, the *Hin*dIII sites of the P1 plasmid replicon (two sites), the P1 lytic replicon (one site), and the pRiA4 replicon (one site) were destroyed sequentially after each component being cloned into the precursor TAC vector by *Hin*dIII digestion, end fill-in, and subsequent religation. The two *Hin*dIII sites of the *sacB* structural gene were destroyed by PCR site-directed mutagenesis. The complete sequence of the vector is available at the DDBJ/EMBL/GenBank accession number AB020028.

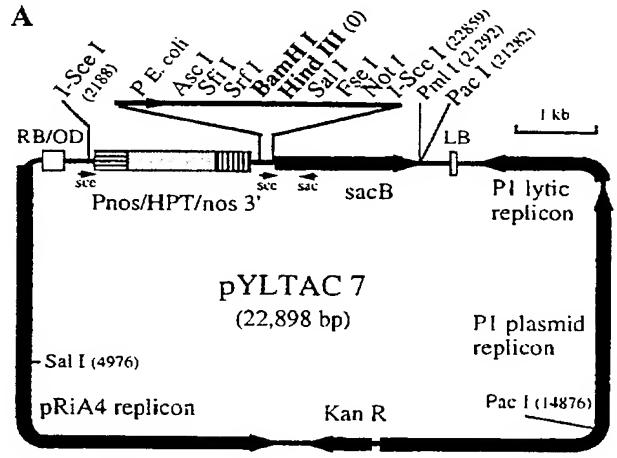
Construction of a TAC Library of *A. thaliana*. Using a nuclei-based method of Liu and Whittier (17), very high molecular weight DNA (>2.5 Mb) was isolated from *A. thaliana* (Columbia ecotype). The DNA was digested partially with *Hin*dIII and size-fractionated in the 75- to 100-kb size range as described (10). The partially digested and size-selected DNA fragments were ligated with *Hin*dIII-digested pYLTAC7 and then used for transformation of *E. coli* DH10B by electroporation. Transformants carrying inserts were selected on LB agar plates containing 25 μg/ml kanamycin and 5% sucrose (4). Details of the library will be published elsewhere.

Plant Transformation. TAC clones were selected randomly for plant transformation from a genomic DNA library of *A. thaliana* ecotype Columbia (unpublished results). TAC clones covering the *SGR1* locus (18, 19) were isolated from the library by using two restriction fragment length polymorphism markers, CDC2B and KSAP3. *E. coli* cells carrying these clones were cultured at 37°C in LB containing 25 μg/ml kanamycin. When the cell density reached an OD_{600} of 0.4–0.8, isopropyl β-D-thiogalactoside was added to a concentration of 0.2 mM and the cells were cultured for an additional 5–12 hr. TAC plasmids were isolated by the alkaline lysis method. The TAC plasmids were introduced into *A. tumefaciens* strains C58C1(MP90), C58C1(GV2260), and EHA105 by electroporation using Gene Pulser (Bio-Rad) with parameters of 100 or 200 ohms and 2.5 kV/0.2 cm. *A. tumefaciens* colonies were selected on LB-agar plates containing 20 μg/ml kanamycin for EHA105, 20 μg/ml of kanamycin and 15 μg/ml gentamycin for MP90, or 25 μg/ml carbenicillin for GV2260. These bacteria were used for transformation of *A. thaliana* plants (3–4 weeks old) of ecotype Wassilewskija (WS) or the *sgr1* mutant plants by the vacuum infiltration method (20) with minor modifications. Transformants (T1 generation) were selected on B5 medium containing 1% sucrose, 15 μg/ml hygromycin, and 250 μg/ml claforan (Hoechst–Roussel). Experiments were carried out by using the progeny of the transformants (T_2 or T_3 plants).

Genomic DNA Analysis. For PCR analysis, plant genomic DNA was prepared on a small scale as described (21). Two primers, CATTACCCTGTTATCCCTA-3′ (sce) and AGGTTTGCAGAACCGGACC-3′ (sac), were used for amplification of the *sacB* gene (see Fig. 1). For Southern analysis of high-molecular-weight genomic DNA, megabase nuclear DNA was prepared from *A. thaliana* plants as described (17). The DNA in low-melting agarose plugs was digested with meganuclease I-*Sce*I (Boehringer Mannheim) and separated on 0.8% agarose gels by field-inversion gel electrophoresis (FIGE) using a PPI-200 power inverter (MJ Research, Cambridge, MA) with programs 3 and 4. The DNA in the gels was irradiated by using UV Stratalinker 2400 (Stratagene) for 5×10^5 J and transferred to Hybond N^+ membranes (Amersham) by alkaline transfer. Southern hybridization was done as described (20) by using the *HPT* gene sequence as a probe.

RESULTS

Design and Construction of the Transformation-Competent Vector. We designed a TAC vector, pYLTAC7 (Fig. 1), to meet the following requirements: (*i*) efficient cloning of large DNA



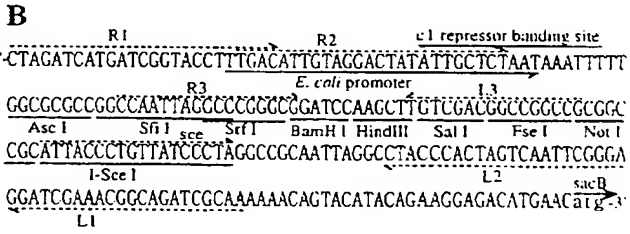


FIG. 1. Physical map of pYLTAC7. (*A*) The map shows the location of some sites for endonucleases that cleave the molecular once or twice. LB and RB, left and right borders, respectively; OD, overdrive sequence; Pnos, promoter of the nopaline synthase gene; *HPT*, coding region of the hygromycin phosphotransferase gene; nos 3′, polyadenylation signals of the nopaline synthase gene; KanR, kanamycin-resistance gene (*NPT1*). The complete sequence of the vector is available in the GenBank database (accession no. AB020028). (*B*) Sequence of the cloning-site region upstream of the *sacB* gene. The primer sets (R1, R2, R3, L1, L2, and L3) are designed for isolation of end fragments of the inserted DNA by thermal asymmetric interlaced PCR (*TAIL-PCR*) (22).

fragments, (*ii*) stable maintenance of inserted fragments in both *E. coli* and *A. tumefaciens*, and (*iii*) competence for transferring inserted DNA into plant genomes via *Agrobacterium*.

It is known that large, foreign DNA fragments are maintained stably in single-copy plasmids such as P1 or BAC (5, 23). We, therefore, used the P1 bacteriophage replicon (23) and the pRiA4 replicon of the Ri plasmid (15), which render the copy number of the plasmid single in *E. coli* and *Agrobacterium*, respectively. In fact, the structural stability of the genomic clones of *Arabidopsis* in P1 (10) and TAC (this study) vectors in *E. coli* has been shown during the genome-sequencing project conducted by Kazusa DNA Research Institute (Chiba, Japan) through analysis of a large number of the clones that cover, in total, 94% of chromosome V of *A. thaliana* (24). The low-yield disadvantage of single-copy plasmids for DNA preparation or library screening is overcome by amplifying the plasmid by releasing the suppresser of the P1 lytic replicon with isopropyl β-D-thiogalactoside (23).

Considering that electroporation is a conventional and efficient technique for transferring large plasmids into *Agrobacterium*, we did not introduce the pRK2 oriT sequence into our TAC vector, which is necessary for delivering plasmids from *E. coli* to *Agrobacterium* by the triparental-mating method (25).

We placed the plant-selectable marker gene (*HPT*), which is driven by the nopaline synthase gene promoter (Pnos), adjacent to the right border rather than to the left border in the vector. Because T-DNA transfer is initiated from the right border (25), transformants selected by the *HPT* gene could carry either the entire or truncated T-DNA. Most transgenes analyzed were not truncated (see below).

Because *Hin*dIII cohesive ends ligate efficiently, we created a unique *Hin*dIII-cloning site in the vector. The vector also has a unique *Bam*HI-cloning site that is suitable for preparing libraries with small (especially <30-kb) *Sau*3AI/*Mbo*I fragments. The *Hin*dIII and *Bam*HI sites were inserted between the *sacB* gene and its promoter. The production of levansucrase encoded by the *sacB* gene in *E. coli* is lethal in the presence of 5% sucrose in agar medium (26). Thus, insert-bearing clones can be selected on sucrose-containing agar plates, leading to a low level of "empty vector" transformants in libraries.

Adjacent to the *Hin*dIII and *Bam*HI sites, five rare-cutter sites (*Asc*I, *Sfi*I, *Srf*I, *Fse*I, and *Not*I) were created, which can be used for preparing nested deletion clones from a large genomic DNA fragment inserted in the vector. Two I-*Sce*I sites flanking the cloning sites and the Pnos sequence were engineered in the vector. With an 18-bp recognition sequence, I-*Sce*I should occur only once in 6.9×10^{10} bp for perfectly random sequence. This design enabled a size check of the entire transferred DNA segment in transgenic plants by probing Southern blots with the Pnos/*HPT* selection marker sequence.

Stability of TAC Clones. By ligating genomic DNA fragments (*ca.* 60–100 kb) of *Arabidopsis* ecotype Columbia into the *Hin*dIII site of the vector and subsequently transforming into *E. coli* DH10B, we constructed a TAC library consisting of *ca.* 10,000 clones (unpublished results). To investigate structural stability of TAC clones in *E. coli* and *A. tumefaciens* strains, 35 *E. coli* clones were selected randomly from the library and plasmids were isolated. The plasmid DNAs were electroporated into *A. tumefaciens* strain C58C1(MP90). Thus passaged, the plasmid DNAs were re-isolated from the *Agrobacterium* transformants and transferred back to *E. coli*. Restriction analysis of these plasmids indicated that 34 clones were maintained completely intact (Fig. 2). One clone in our experiments was found to be unstable in *A. tumefaciens*. Therefore, we recommend checking the stability of each TAC clone in *A. tumefaciens* before plant transformation.

Transformation of *A. thaliana* with TAC Clones. To assess the transformation efficiency with respect to T-DNA sizes, we conducted plant transformation by using TAC clones with or without a large genomic DNA insert. TAC clone 20D10 (the same clone shown in Fig. 4*A*) carries a *ca.* 80-kb insert of *A. thaliana* genomic DNA. This clone and the vector alone, which contains a T-DNA of 4.4 kb between the right and left borders, were introduced into *A. tumefaciens* strain C58C1(MP90). These plasmids were maintained stably in the host cells. We transformed *Arabidopsis* ecotype WS by using these bacteria by the vacuum-infiltration protocol. From three independent experiments, we obtained 482 hygromycin-resistant plants from 142 plants treated with the 20D10 bacterium and 688 hygromycin-resistant plants from 127 plants treated with the bacterium carrying the vector alone (Table 1). These results indicate that the transformation efficiency is not affected substantially by the sizes of introduced T-DNA within this range. This efficiency is comparable to those we obtained by using other binary vectors such as pBI121. Transformants also were obtained from the Columbia ecotype with other TAC clones (see Table 2 and data not shown). Our experiments showed that *A. tumefaciens* strains C58C1(GV2260) and EHA105 also were able to transform *Arabidopsis* with lower but sufficient efficiencies (data not shown).

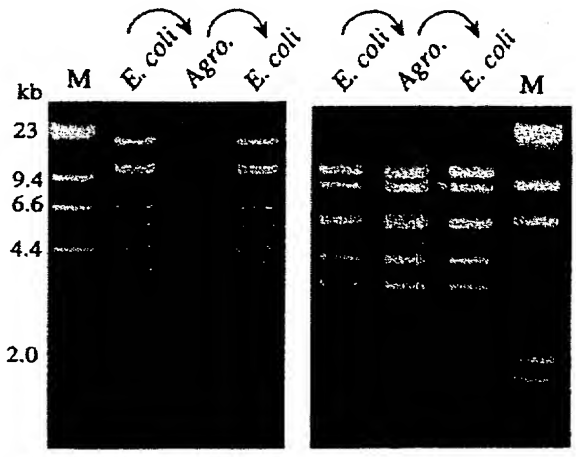


FIG. 2. Stability of two TAC clones in *E. coli* and *A. tumefaciens*. TAC plasmid DNA of two independent clones were isolated from *E. coli* (DH10B) and were used for transformation of *A. tumefaciens* C58C1(MP90). The plasmid DNA in the *Agrobacterium* host was transferred back to the *E. coli* host. Digestion of plasmid DNA in each step is shown in this figure. (*Left*) A TAC clone digested by *Hin*dIII. (*Right*) Another TAC clone digested by *Pst*I. M, molecular markers.

Analysis of Transgenic Plants. To examine whether large T-DNAs were integrated in their entirety into the plant genome, we analyzed the transferred segments by PCR and

Table 1. Transformation efficiency of *A. thaliana* with TAC clones

Exp.	Construct	Transformants/ treated plants	Seeds screened/ transformant
1	1	115/46 (2.5)	770
	2	265/43 (6.2)	404
2	1	268/48 (5.6)	328
	2	325/36 (9.0)	246
3	1	99/48 (2.0)	970
	2	98/48 (2.0)	876
Sum (1 + 2 + 3)	1	482/142 (3.4)	565
	2	688/127 (5.4)	397

A TAC clone having an insert of 80 kb (construct 1) and the TAC vector without an insert (construct 2) were used for transformation of *A. thaliana* ecotype Wassilewskija. Numbers of parentheses are transformation efficiency. The results of three independent experiments and their sum are shown.

Table 2. Complementation of the *A. thaliana sgr1* mutant with TAC clones carrying the *SGR1* gene

T1 line	Clone	Complementation	Segregation in T_2 family for hygromycin resistance (R:S)	χ^2 (3:1)
A	5I12	Yes	139:50	0.213
B	5I12	No	94:29	0.133
C	5I12	Yes	166:63	0.770
D	5I12	Yes	169:44	2.142
E	5I12	Yes	215:64	0.632
F	5I12	Yes	221:94	3.938*
G	5I12	Yes	199:25	22.88**
H	20D10	Yes	201:81	2.085
I	20D10	Yes	208:76	0.469

*,**, Significant at the 5% and 1% levels, respectively.

genomic Southern blotting. The *sacB* gene sequence, located near the left border, was amplified from 32 of 36 hygromycin-resistant plants obtained by using four distinct TAC clones carrying 40- to 80-kb inserts (Fig. 3*A*). This result suggests that in most transformants the entire inserts were transferred to the *Arabidopsis* genome.

The genomic DNAs of transformants that were created by using a TAC clone, 5I12, carrying a 75-kb insert (see below) were digested in agar plugs by I-*Sce*I and subjected to Southern blotting experiments by using the *HPT* gene as a probe. The results showed that the sizes of the I-*Sce*I fragments of three transformants were identical to those of the original plasmids (Fig. 3*B*, lanes 3, 5, and 6), indicating perfect, intact transfer of the 75-kb insert. However, one transgenic line (Fig. 3*B*, lane 4) did not show the I-*Sce*I fragment, indicating the loss of at least one of the I-*Sce*I sites.

The I-*Sce*I fragment of 45 kb detected in a T_2-generation transgenic line was inherited faithfully in the T_3 generation (Fig. 3*C*).

Physical Mapping and Complementation of the Gravitropic Mutant *sgr1*. We tested the usefulness of the TAC system for positional cloning with the *SGR1* gene as a model. The *Arabidopsis* mutant *sgr1* is deficient in the gravitropic response of its hypocotyl and stem (17). The *SGR1* locus has been mapped on chromosome III with two adjacent DNA markers, CDC2B and KSAP3 (18). A TAC contig covering the locus was constructed by screening the TAC library using these DNA markers (Fig. 4*A*). While this study was underway, we noted that the *SGR1* was allelic to the *SCARECROW* gene, which had been isolated as the gene for the radial organization of the *Arabidopsis* root (27). Therefore, we tested our contig by PCR with primers specific to the *SCARECROW* gene and identified three positive TAC clones. Two of these clones, 5I12 (*ca.* 75 kb) and 20D10 (*ca.* 80 kb), were introduced into the mutant plant *sgr1* (ecotype Columbia background) by vacuum infiltration using *A. tumefaciens* strain C58C1(MP90). Of nine transgenic T1 plants, eight plants recovered the normal gravitropic response (Table 2). Line B appears to have a deletion in the transferred insert (Fig. 3*B*, lane 4). In the T_2 generation of line E grown on medium without hygromycin, a 3:1 ratio of wild type to mutant was observed (Fig. 4*B*), as expected if the T_1 generation carried the complementing DNA as a single-locus, hemizygous insertion. In fact, all hygromycin-resistant T_2 plants in these eight lines exhibited wild-type gravitropism in shoot growth. These results indicate that the transgene *SGR1* is expressed normally in the transgenic progeny plants, complementing the *sgr1* mutation.

The results also demonstrate a single locus insertion of the transgene in all but one (line G) of the T_2 families (Table 2) because segregation of the hygromycin-resistant phenotype of the transgenic lines was consistent with 3:1 segregation.

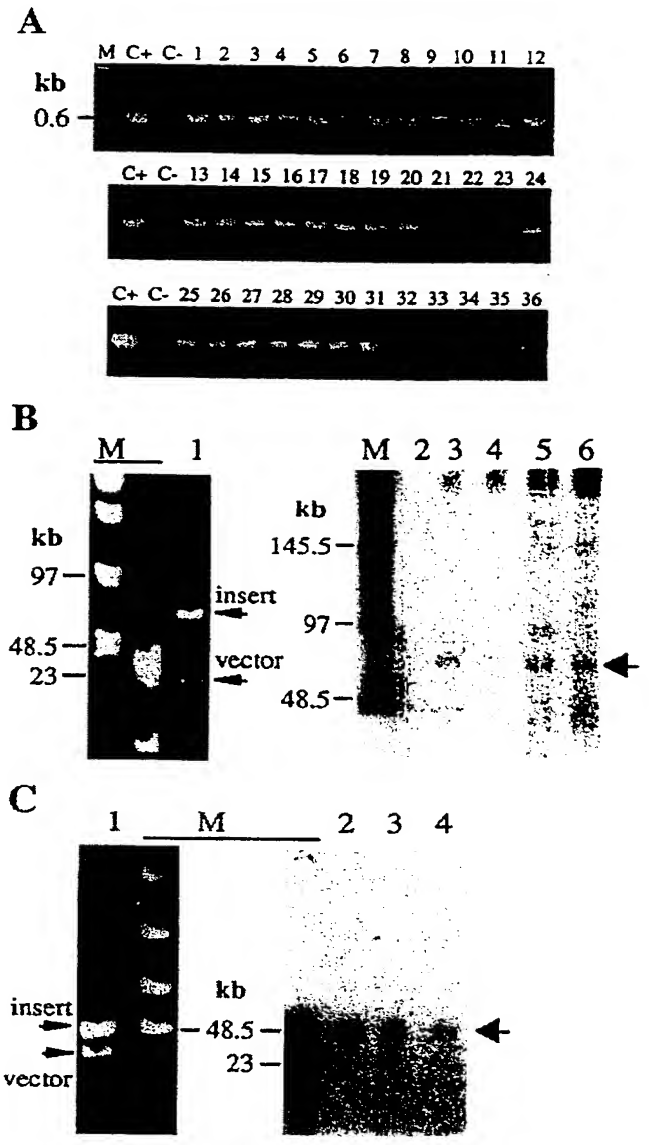


FIG. 3. Transgenes in *Arabidopsis* plants transformed with TAC clones. (*A*) *A. thaliana* ecotype WS was transformed with TAC clones carrying either 40-kb (lanes 1–20) or 80-kb (lanes 21–36) genomic DNA fragments of ecotype Columbia. The *sacB* gene of 36 transgenic plants (hygromycin-resistant plants) was checked by PCR (Fig. 1). C+ and C−, positive (a TAC clone) and negative (untransformed plant) controls, respectively. (*B*) Transgenic lines transformed with a 75-kb TAC clone was self-crossed, and then the resulting T_2 plants were analyzed by genomic Southern experiments. Genomic DNAs of transgenic and untransformed (negative control) plants were digested with I-*Sce*I and hybridized with a *HPT* gene probe. The hybridized bands (lanes 3, 5, and 6) are shown by the arrow on the right. No hybridization band corresponding to the I-*Sce*I fragment is seen in lanes 2 and 4. Lane 1, plasmid DNA digested with I-*Sce*I; lanes 2–6, genomic Southern blotting of DNAs from untransformed plants (lane 2) and T_2 lines (lanes 3–6). (*C*) The progenies of a transgenic line transformed with a 45-kb TAC clone were analyzed. Genomic DNAs of a T_2 line (lane 2) and its T_3 progenies (lanes 3 and 4) were digested with I-*Sce*I and hybridized with the *HPT* gene probe. Lane 1, plasmid DNA digested with I-*Sce*I; lanes 2–4, genomic Southern blotting of DNA digested with I-*Sce*I.

DISCUSSION

A major drawback of positional cloning is its dependence on detailed genetic analysis involving many progeny to achieve

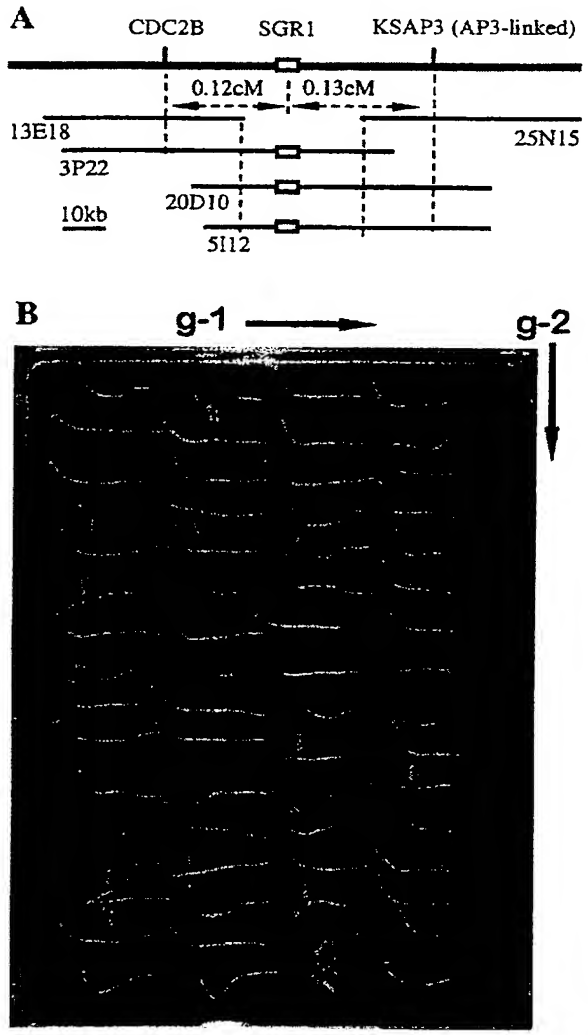


FIG. 4. Complementation of the *sgr1* mutation with large TAC clones of wild type. (*A*) The *SGR1* locus was covered contiguously by TAC clones (13E18, 3P22, 20D10, 5I12, and 25N15) carrying large (*ca.* 80-kb) genomic DNA fragments of *A. thaliana* Columbia ecotype that were isolated by using two DNA markers, CDC2B and KSAP3. (*B*) Segregation of the T_2 family seedlings of the transformed line E for gravitropic responses. Seedlings were grown in darkness for 3 days after germination with the plate setting as the direction of gravity indicated by g-1. The plate then was turned by 90° as indicated by g-2, and the seedlings were grown in darkness for 24 hr. About 75% of seedlings showed distinct negative gravitropic curvature in hypocotyls as wild type, whereas the remaining (marked by arrows) did not show gravitropic curvature at all (*sgr1* mutation phenotype).

sufficiently fine mapping. Therefore, genetic mapping is a rate-limiting step in positional cloning. To accelerate this process for plants, we developed a TAC vector, pYLTAC7. This vector is suitable for stable maintenance of large genomic DNA fragments in both *E. coli* and *A. tumefaciens* and is competent for transfer of insert DNA into a plant genome by *Agrobacterium*-mediated transformation. To demonstrate this system's practical utility in physical mapping and complementation, we complemented a *sgr1* mutant of *A. thaliana* with large insert (*ca.* 80 kb) TAC clones carrying the wild-type allele. Recently, positional-cloning approaches have come to be preferred over DNA tagging for isolation of *Arabidopsis* genes identified through mutation (1). Although T-DNA and transposon tagging have been used for isolation of many plant genes, this approach has severe limitations because of the null function of most tagged alleles. It is difficult to dissect pathway interactions or processes vital for cell maintenance, especially any processes required after meiotic cell division. In contrast, chemical mutagens such as ethyl methanesulfonate can generate not only null mutants, but also mutants with partial or conditional gene function such as temperature-sensitive mutants. Analyses of these mutants and isolation of the genes defined by these mutations become desirable for understanding complex biological processes. In fact, the *filamentous flower* gene that is involved in flower development of *A. thaliana* was isolated successfully by using the TAC cloning system (S. Sawa and K. Okada, personal communication). A cell-wall synthesis gene that is defined by a temperature-sensitive mutation also was isolated by using this system (S. Sato, T. Kato, and D.S., unpublished results).

The TAC system is especially useful for positional cloning of genes when the position itself is imprecisely known. Mutations may exhibit incomplete penetrance whereby the mutant phenotype is subtle or depends on additional factors such as the external environment or other genetic loci. In this situation, perfect scoring of mapping crosses may not be achieved (e.g., *cer9*) (10). For the same reason, the map positions determined for quantitative trait loci (QTLs) are also approximate. Because QTLs control such important agronomic properties as yield, disease resistance, and stress tolerance, cloning of these elusive genes is a high economic priority. Genes located in recombination cold-spot regions (7) are also hard to be isolated by positional cloning. Thus, either scoring uncertainty or a scarcity of informative recombinational events can limit researchers' ability to narrow a gene's chromosomal position. TAC-based contigs can cover relatively wide chromosome regions with just a few clones. Thus, less mapping precision is required to make complementation testing feasible. Once a complementing clone is identified, the five rare-cutter restriction sites adjacent to the cloning site of TAC vector pYLTAC7 facilitate creation of nested deletion clones for further narrowing the complementing gene.

Elucidating the molecular genetics of *Arabidopsis* will be accelerated by using the TAC clones. At present, the genome mapping and sequencing project of *A. thaliana* at Kazusa DNA Research Institute (Chiba, Japan) has been using the TAC library for preparing contigs and sequencing parts of chromosomes V and III (24, 28, 29). The combination of TAC clone-based physical maps and their genome sequence data will greatly facilitate assignment and confirmation of gene function in *Arabidopsis*. The complete *Arabidopsis* genome sequence will be determined within a few years (2). With this in mind, sequencing of both ends of more than 2,000 TAC clones would suffice to identify clones covering nearly the entire genome of *Arabidopsis*. The TAC library of *Arabidopsis* will be distributed to academic researchers through the *Arabidopsis* Biological Resource Center at The Ohio State University.

Our results demonstrated high transformation efficiency with TAC clones; more than 1,000 transformants were obtained from three small-scale transformation experiments (Table 1). The *virG*- and *virE*-carrying helper plasmids used in the binary-BAC system for enhancing large T-DNA transfer (11) are not necessary for efficient transfer of 80-kb (this study) or larger TAC inserts. Among the transformants tested, most carry the entire T-DNA as confirmed by PCR and Southern analysis (Fig. 3*B*).

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Analysis of the molecular basis of vernalization in *Arabidopsis thaliana*

Allison Wilson and Caroline Dean

The Arabidopsis genes FCA *and* FRI *are being studied to dissect the molecular basis of the vernalization requirement in plants. Recessive mutations in* FCA *and dominant alleles of* FRI *cause late flowering. The late flowering phenotype can be converted to early flowering by vernalization in both cases. The* FCA *gene encodes a protein containing RNA-binding domains, suggesting* FCA *plays a role in post-transcriptional regulation. Flowering time and vernalization response have been analysed with* fca *in different mutant backgrounds. The* fca *mutant has also been the starting point for a second round of mutagenesis to identify genes necessary for the vernalization response.*

Key words: *Arabidopsis* / flowering time / genes / mutants / vernalization

In plants, successful fertilization and seed formation depend on the correct timing of the switch from vegetative to reproductive development at the shoot apex. The molecular and genetic advantages of *Arabidopsis thaliana* (Arabidopsis) [1,2] make it a useful model system for analysing floral induction and more than 22 loci have been identified that affect flowering time in Arabidopsis (for recent reviews see refs 3,4). These loci have been placed into various phenotypic groups depending on the response of mutants to environmental conditions. Arabidopsis plants with recessive late flowering mutations at the loci *FCA*, *FPA*, *FVE*, *FY*, or *LD* require vernalization for early flowering and show a late flowering phenotype in both long (LD) and short days (SD) photoperiods. These loci have been classified as acting within a constitutive floral promotion pathway. Plants with late flowering mutations at other loci (*CO*, *FD*, *FE*, *FHA*, *FT*, *FWA*, and *GI*) show less sensitivity to photoperiod or vernalization, and are thought to be involved in a separate, LD-dependent, floral promotion pathway.[4]

Analysis of loci causing late flowering in natural populations has revealed additional loci having major effects on flowering time. Arabidopsis plants containing dominant late flowering alleles at both the *FRI* and *FLC* loci are late flowering and can be reverted to early flowering by a vernalization treatment.[5–8] In the wild and in agriculture, vernalization plays a major role in synchronizing flowering in populations, however the nature of the cold induction process is poorly understood.

This review first discusses two loci involved in the vernalization requirement, *FCA* and *FRI*, and then describes the identification and characterization of *VRN1* and *VRN2*, loci involved in the response to vernalization.

Loci involved in the vernalization requirement: *FCA* and *FRI*

The studies of *FCA* described in this review all involve *fca-1*, which was isolated as a late flowering EMS-induced mutation in the normally early flowering Landsberg *erecta* (L*er*) background.[9] The *FRI* alleles described in this review are the dominant *fri-1* allele, originally named *F*[7,10] and the dominant allele in line H51,[6] isolated from the late flowering Stockholm ecotype, which we have designated *fri-2*. A comparison of the flowering times, as assayed by leaf number, in *fca-1* and a line H51, homozygous for the dominant *fri-2* allele are shown in Table 1. Plants homozygous for the *fca-1* mutation or carrying at least one allele of *fri-2* flower significantly earlier after a vernalization treatment (Table 1).

Genetic interactions between *fca-1* and other loci

In order to analyse interacting pathways, double mutants have been made between *fca-1* and mutations thought to be involved in the transition to flowering (J. Chandler, T. Page and C. Dean, unpublished). It

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Table 1. Flowering time, as assayed by leaf number, of various *Arabidopsis* genotypes, with and without vernalization treatment. For Li-5 and H51 (ref 6), leaf numbers are rosette leaves only. For all other genotypes, total leaf number (rosette + cauline) is recorded (J. Chandler and C. Dean, unpublished data). The *fca-1*, *vrn1-1 fca-1* and *vrn2-1 fca-1* mutations are in the L*er* background. Numbers are means of at least 10 plants ± standard error. Vernalization treatment was for 8 weeks at 4°C

Genotype	– vernalization	+ vernalization
L*er*	6.1±0.1	5.9±0.1
fca-1	27.2±1.0	8.1±0.3
Li-5	9.8±0.2	11.7±0.2
H51 (homozygous for *fri-2*)	32.5±0.7	10.3±0.2
vrn1-1 fca-1	29.3±1.6	17.0±0.9
vrn2-1 fca-1	46.1±2.6	31.8±1.8

has been suggested that gibberellins (GA) can regulate the floral transition and that they play a role in the vernalization response (refs 11, 12, for reviews see refs 3, 4). Often the action of GA is antagonized by abscisic acid (ABA). In support of this, *aba-1* (ABA deficient) and *abi1* (ABA insensitive) mutants flower slightly early in SD[13] while *gai* (a gibberellin insensitive mutant) and *ga1-3* (a GA deficient mutant) cause late flowering in SD.[12] A strong interaction between *fca-1* and *ga1-3* was observed with the double mutant flowering much later than *fca-1* (J. Chandler and C. Dean unpublished). In contrast, the *gai*, *fca-1* double mutant flowered earlier than *fca-1*. Vernalization of the *fca-1*, *ga1-3* double mutant reduced the flowering time to that of the *ga1-3* parent (J. Chandler and C. Dean, unpublished). One interpretation of this result is that *FCA*, *GA1* and vernalization act in different pathways to regulate flowering time. Double mutant analysis with *fca-1* and *abi1*, *abi2*, *abi3* suggests that the ability of vernalization to promote flowering is independent of ABA as controlled by *ABI1*, *ABI2* and *ABI3* (J. Chandler and C. Dean, unpublished).

Genes controlling flowering time must, either directly or indirectly, regulate genes involved in meristem identity such as *TFL*, *LFY* and *API*. Recent data indicates that the late flowering loci of Arabidopsis influence the length of all phases of development: juvenile and mature vegetative, inflorescence and floral.[14] One model suggests that environmental factors and genes regulating phase change, including the late flowering loci, do so by influencing the total level of an undefined activity called controller of phase switching (COPS). As COPS activity decreases to various levels, different developmental programs (vegetative, inflorescence and finally flowering) are activated (reviewed in ref 3). Analysis of double mutants between *fca-1* and *tfl2*, *lfy-5* and *ap1-1* showed that the phenotype of the double mutant combinations grown in long days was similar to the phenotype of the single *tfl2*, *lfy-5* and *ap1-1* mutations grown in SD (T. Page and C. Dean, unpublished). The *fca-1*,*lfy-5* double mutant was the only combination where the phenotype of the double in long days could be distinguished from the phenotype of the single meristem identity mutant in SD, with the latter being more extreme. The phenotypes of *tfl2*, *lfy-5* and *ap1-1* double mutant combinations with *fca-1* were indistinguishable from *tfl2*, *lfy-5* and *ap1-1* double mutants with *co-2* (a late flowering mutation in the LD dependent floral promotion pathway — see R. Simon and G. Coupland, this issue).

These data suggest that the delay in flowering imposed by late flowering mutations and SD photoperiods are both due to an indirect effect on meristem identity genes.

Molecular analysis of *FCA*

In order to dissect the role of the *FCA* gene product in regulating the floral transition, the *FCA* locus was isolated using map-based cloning techniques (I. Bancroft, C. Dean, unpublished). *FCA* was mapped between RFLP markers m226 and m580 on chromosome 4, and cosmid clones spanning the *FCA* region were isolated from a L*er* library. Cosmids containing the *FCA* gene were identified by their ability to complement the fca phenotype, and were used to identify putative *FCA* transcripts by hybridization to a cDNA library prepared from mixed RNA samples.[15] Two partial cDNA clones were isolated, representing two of the three transcripts produced from alternative splicing of the *FCA* gene. The multiple transcripts are present in all developmental stages and tissues so far examined.

The *FCA* gene is 8.1 kb long, contains 20 exons and has a 5' untranslated leader of approximately 300 bp. It encodes a protein containing two RNA-binding domains, characteristic of proteins involved in RNA processing (R. Macknight and C. Dean, unpublished). The C-terminal region of the *FCA* protein is glutamine rich and carries a short amino acid sequence showing strong homology to a *C. elegans* and a *S. cerevisiae* EST, both of unknown function.

The presence of RNA binding motifs in the *FCA* protein suggests it might post-transcriptionally regulate either the splicing or the transcript levels of other genes involved in floral induction or floral

meristem identity. A well known example of a developmental pathway acting through regulation of splice site choice is the sex determination of *Drosophila* (reviewed in ref 16). One gene in this pathway, *SEX-LETHAL* (*SXL*), controls 3' splice site selection in its own transcript and in a downstream gene, *TRA2*. Splice site selection determines whether the fly is male (a truncated protein is made) or female (a full length protein is made). Future experiments will determine whether *FCA* can regulate the splicing or abundance of its own transcript and/or transcripts of genes thought to be downstream of *FCA* in the floral induction pathway (R. Macknight and C. Dean, unpublished).

It will be interesting to see whether *FCA*-related sequences in Arabidopsis and other plant species correspond to genes known to be involved in regulating flowering time. Low stringency hybridization experiments reveal that *FCA* is part of a small gene family in *Arabidopsis* and that *FCA*-related genes are present in all plant species analysed, including a range of dicots and monocots. The *Brassica napus FCA* gene has been partially analysed and comparison with Arabidopsis *FCA* shows a high degree of conservation at the amino acid level, especially over the RNA binding motifs (R. Macknight and C. Dean, unpublished). Additional copies of the *VRN1* gene in wheat promote a spring habit, ie. reduce the vernalization requirement [17], a situation mimicked by dominant alleles at the *FCA* locus. Cloning and mapping of *FCA* homologues from a cereal genome will make it possible to determine whether they correspond to any of the mapped *VRN* loci from wheat.

Genetic analysis of *FRI*

In naturally occurring ecotypes, late flowering is conferred by dominant alleles at the *FRI* locus in all crosses so far examined.[5-8] Extreme lateness in plants containing a dominant *FRI* allele requires the presence of another dominant allele at a second locus, *FLC*.[7,18] Dominant *FLC* alleles have been found in all *Arabidopsis* ecotypes analysed so far, except for L*er* and C24.[19] L*er* has a recessive allele of *FLC* that can suppress the late flowering phenotype conferred by dominant *FRI* alleles.[7,18]

The *F* mutant[10] was shown to be a probable allele of *FRI* and was renamed *fri-1*.[7] In addition to flowering late, *fri-1* mutants have poorly developed main inflorescences, with vegetative rosettes forming at the site of axillary buds at early inflorescence nodes. Vernalization completely suppresses the *fri-1* phenotype, so that vernalized plants look like wild-type. The other dominant *FRI* alleles do not show this extension of vegetative growth in the axillary buds.

It is interesting to speculate how two dominant alleles at a single locus, *fri-1* and *fri-2*, could give similar yet not identical phenotypes, *fri-1* being more extreme. It has been suggested that the *FRI* gene encodes a protein involved in repression of flowering and/or promotion of vegetative development.[8] Differences in the amount, timing, or location of *FRI* expression could result in the flowering time variation seen in naturally occurring ecotypes. The dominance of the *fri-1* mutation might be explained by a rearrangement in the gene (possibly the promoter) that further increases the level of *FRI* or changes its expression pattern. In *fri-1* mutants the apical meristem is able to produce a main inflorescence. This suggests that the effects of *fri-1* are stronger in axillary meristems and may indicate that *FRI* is expressed differently in apical versus axillary meristems, or that the two types of meristems show different sensitivities to the *FRI* product. Other researchers have noted differences in fates between lateral and apical meristems.[20-22]

Cloning of the *FRI* locus will allow some of the models about its function and expression to be tested. The *FRI* locus has been mapped to chromosome 4, in a cross between H51 (a late flowering line homozygous for the *fri-2* allele) and Li-5 (an early flowering line).[6] The *fri-2* mutation co-segregates with RFLP markers g3843, mi204 and mi51, and this region is completely covered by a physical map built in yeast artificial chromosome (YAC) clones.[23] Fine mapping experiments prior to cloning are now underway (J. West and C. Dean, unpublished).

Vernalization requirement versus vernalization response

While dominant alleles at *FRI* and recessive alleles at *FCA* cause plants to require a vernalization treatment for early flowering, it is unlikely that *FRI* and *FCA* are actually components of the vernalization response pathway itself. Most probably, vernalization acts through an alternative pathway that promotes flowering and makes the constitutive flowering pathway, including *FCA*, unnecessary. This alternative vernalization pathway may be able to inactivate or bypass the vegetative promoter/floral inhibitor thought to be produced by *FRI*.[8] In order to understand how

vernalization acts to suppress late flowering in *fca-1* and *fri-1* plants, it will be necessary to identify and clone genes involved directly in the perception and response to vernalization.

Figure 1 summarizes some of the characteristics of the vernalization response that have been deduced from physiological experiments carried out on various plant species (reviewed in refs 24-26). It is important to note that cold temperature treatment has a number of effects on plant development in addition to its effect on flowering time. Many plant species have evolved dormancy mechanisms to avoid precocious germination, and cold temperature relieves this dormancy in a process known as stratification. In addition, many species exhibit a cold acclimation response so that they are able to tolerate freezing conditions if they have first experienced 3–5 days of non-freezing cold temperature (reviewed in ref 27). Whether acclimation, stratification and vernalization are sensed by similar kinds of mechanisms remains an open and interesting question, and one that the isolation of mutations in the vernalization response will help to address.

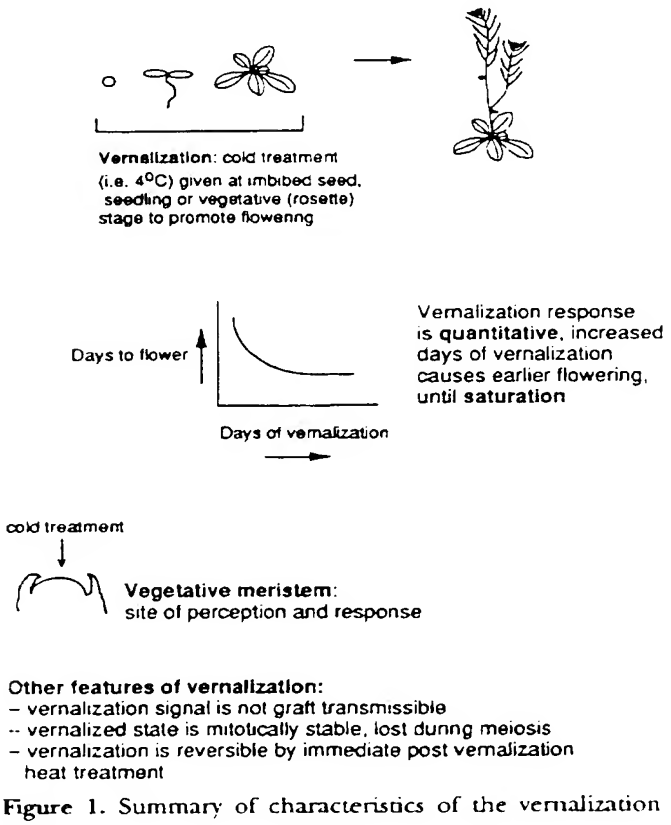

Figure 1. Summary of characteristics of the vernalization response.

Identification of genes involved in perceiving or responding to vernalization

In order to identify mutations in the vernalization response pathway, mutagenized *fca-1* plants were screened for plants that flowered late after vernalization treatment. Plants homozygous for the *fca-1* mutation were used for mutagenesis because L*er fca-1* represents a well characterized genetic background that exhibits a strong vernalization response. Mutations isolated in the L*er fca-1* background can be mapped using RFLP analysis and can be crossed to other mutations isolated in the L*er* background to look for genetic interactions.

A *vrn* mutation, defined as a mutation that causes late flowering by disrupting the vernalization response of L*er fca-1* plants, should fit several criteria. Unvernalized *vrn fca-1* plants should flower at the same time as *fca-1*, while vernalized *vrn fca-1* should flower later than *fca-1* plants, due to disruption of the vernalization response that would normally suppress the late flowering *fca-1* phenotype. These *vrn* mutations differ from mutations in LD-dependent flowering pathway (ie. *co*) which cause plants to flower late with or without vernalization treatment, and which would also be picked up in such a screen. Figure 2 illustrates the strategy used to identify putative *vrn* mutants and to screen out mutations in the LD-dependent flowering pathway.

Screens of both EMS and gamma mutagenized lines have identified various potential *vrn* mutations that represent at least 3 independent loci (J. Chandler, A. Wilson, Y. Levy and C. Dean, unpublished results). The two best characterized mutations have been designated *vrn1-1* and *vrn2-1* (J. Chandler, A. Wilson, and C. Dean, manuscript in preparation).

Analysis of *VRN1*

The *VRN1* locus is represented by a single recessive allele, *vrn1-1*. The *vrn1-1 fca-1* double mutants flower later than *fca-1* plants after a vernalization treatment, but at the same time as *fca-1* without vernalization (Table 1). The *vrn1-1* mutation was recombined away from *fca-1* and re-isolated in the L*er* background on the basis of its ability to disrupt the vernalization response exhibited by L*er* plants grown in SD. Double mutants made by crossing *vrn1-1* to other late flowering mutants also showed a decreased vernalization response. These results suggest that *vrn1-1* is a mutation that specifically disrupts the vernalization

pathway of *Arabidopsis* and not a mutation in the LD-dependent flowering pathway or a mutation that interacts specifically with *fca-1*.

It was shown that *vrn1-1 fca-1* plants have a normal acclimation response (J. Martinez-Zapater, unpublished) and RNA blot analysis showed that the cold induction of the COR15 gene[28] was not altered in *vrn1-1 fca-1* plants (J. Chandler and C. Dean, unpublished). Thus, the *vrn1-1* mutation seems to alter the vernalization response without disrupting other cold responses exhibited by *Arabidopsis*.

To facilitate cloning of the *VRN1* locus, *vrn1-1* has been mapped using RFLP markers to chromosome 3, approximately 1 cM from mi339 (J. Chandler, A. Wilson and C. Dean, manuscript in preparation) on the recombinant inbred map.[29] A targetted tagging screen has been set up to tag the *VRN1* locus using a linked *Ds* element so that the tagged *VRN1* gene can be cloned by IPCR methods (A. Wilson, T. Page, Y. Levy and C. Dean, unpublished). Putative *vrn1* mutants isolated in the screen are being tested for linkage to a *Ds* element and for allelism to *vrn1-1* (Y. Levy and C. Dean, unpublished).

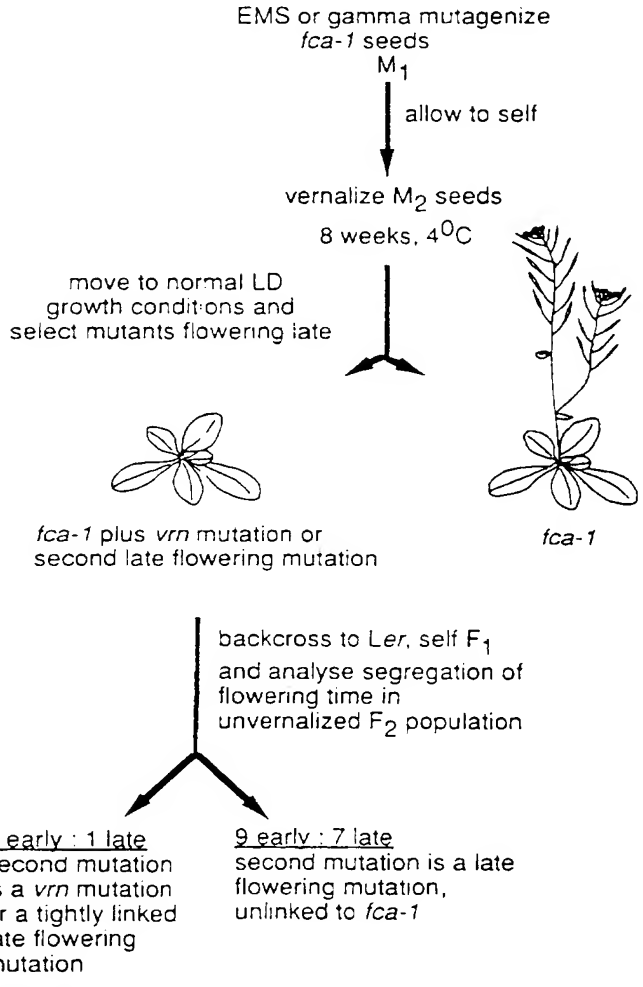

Figure 2. Screen used to isolate mutations in the vernalization response pathway of *Arabidopsis*.

Analysis of *VRN2*

The recessive *vrn2-1* mutation was also isolated by the screening methods described in Figure 2. However, further studies indicated that unvernalized *vrn2-1 fca-1* plants flower later than *fca-1* controls, and RFLP mapping of *vrn2-1* showed that it is linked to *fca-1* on chromosome 4 (A. Wilson and C. Dean, unpublished). In order to distinguish *vrn* mutations from LD-dependent late flowering mutations, the screen described in Figure 2 relies on the fact that the second mutation is unlinked to *fca-1*. We have made use of transformants carrying the wild-type *FCA* gene in different genomic locations to determine whether *vrn2-1* has a late flowering phenotype in the absence of the *fca-1* mutation and vernalization (A. Wilson and C. Dean, unpublished). Our current model is that *VRN2* promotes flowering to a small extent when plants have not been vernalized, but that its role is significantly increased after vernalization.

The location of *vrn2-1* on chromosome 4 in a region that has been completely covered by YAC contigs[28] will facilitate its cloning by chromosome walking techniques. Fine mapping experiments are underway to localize *vrn2-1* to a region that can be covered by cosmid clones (A. Wilson and C. Dean, unpublished), and complementation of the *vrn2-1 fca-1* phenotype will be used to identify DNA containing the *VRN2* locus.

Conclusion

Clearly our understanding of genes involved in the requirement for vernalization and those necessary for the vernalization response itself is at a preliminary stage. As there appear to be many different signals and pathways involved in the regulation of the transition from vegetative to floral development, it may require the cloning of a number of genes in each pathway before the nature of the regulation becomes clear and before the order of genes in each pathway can be determined. Given the similarity in phenotype

between late flowering mutations, double mutant analysis is not an especially useful way to order genes within pathways. It may prove more useful to look at the levels, types, or patterns of expression of RNA and protein products of one flowering gene in background of different late flowering mutants.

A complete understanding of vernalization and flowering time will depend on the identification of genes directly involved in the perception of the cold treatment, on understanding interactions within and between pathways, and finally it will be crucial to understand how all of the pathways regulating flowering time converge to actually cause the transition of the apex from vegetative to floral growth. The cloning and molecular analysis of flowering genes such as *FRI*, *VRN1* and *VRN2* will be a useful first step in understanding the requirement for vernalization and the vernalization response in *Arabidopsis*.

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